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14. ABSTRACT –

Examination of the enzyme structure for acetylcholinesterase (AChE) reveals two sites of ligand interaction: The peripheral site (P-site) located at the entrance of the gorge, and the acylation site (A-site) at the base of the gorge. Our goal is to develop high affinity cyclic peptide ligands specific for the P-site that would block the access of organophosphate agents while allowing the passage of acetylcholine to the A-site for use by personnel at risk for nerve gas exposure. Our immediate strategy involves the covalent tethering of cyclic inhibitors via a methanethiosulfonate (MTS) linkage to a cysteine on the AChE mutant, H287C. The modified AChEs linked to candidate peptides that inhibit P-site access are selected by affinity chromatography and tested for P-site and A-site affinity by measuring competitive inhibition constants KI2 for propidium and tacrine, inhibitors specific for the P- and A-sites, respectively. We are using a combinatorial approach to identify tethered cyclic peptides with high affinity for the P-site. A cyclic octapeptide library with 3000 compounds was synthesized in 30 subsets. One initial subset of 100 compounds has been screened by covalent tethering, affinity chromatography selection, and analysis of competitive inhibition by ligands that bind to the P-site. Candidate cyclic peptides were released from modified AChEs with substantial P-site blockade by reduction with dithiothreitol, and mass spectrometry (MS) techniques were used to characterize the peptides. MALDI TOF MS revealed a mixture of peptides by detecting a series of predicted peptide masses. Peptide sequences were obtained on an ESI ion trap mass spectrometer with MSn capabilities by following peptide fragments through several stages of consecutive collisionally activated decomposition (CAD) mass spectra. Two peptide masses were selected from this first screen, corresponding to sixteen individual peptides because of several D- and L-amino acid combinations. These peptides are now being screened to identify the individual peptides with the highest P-site blocking activities.

15. SUBJECT TERMS
Acetylcholinesterase, organophosphate, cyclic peptide, catalytic Mechanism, peripheral site, steric blockade, substrate inhibition

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INTRODUCTION

Our studies focus on the enzyme acetylcholinesterase (AChE), which is responsible for hydrolyzing the neurotransmitter acetylcholine (ACh). AChE is a key enzyme at the neuromuscular junction with one of the fastest known enzymatic rates (*1*). X-ray crystallography of this enzyme reveals a large active site gorge made up of two distinct sites separated by a 20Å narrow channel. At the base of this gorge is the acylation site (A-site), while close to the mouth of the gorge is the peripheral site (P-site) (*2*). The presence of two enzymatic sites allows for binding of ligands to either the A-site or P-site as well as to both sites simultaneously. AChE is inactivated by organophosphates (OPs) in pesticides and chemical warfare agents because OPs can pass through the P-site and phosphorylate the catalytic serine in the A-site. Our goal has been to focus on the P-site to design inhibitors that would selectively inhibit OP inactivation of AChE while still allowing hydrolysis of ACh at the A-site.

In recent years we have found that ligands that bind to the P-site inhibit AChE through a process we call *steric blockade* (*3, 4*). This process is manifested as a decrease in the rate constants with which substrates and their hydrolysis products enter and exit the A-site. The concept of steric blockade has led us to a new strategy for the design of drugs to protect AChE from inactivation by OPs. Such a drug must exclude OPs from the A-site while interfering minimally with ACh passage. Our search for a prototype of this drug has focused on cyclic peptide and pseudopeptide compounds, as they have a number of advantages. First, a cyclic molecule is a ring with a pore that in theory can be designed to exclude passage of bulky OPs while allowing smaller ACh to pass. Relatively small cyclic compounds consisting of 6 to 8 amino acids possess the necessary pore diameter to permit passage of ACh. Second, the incorporation of both natural and unnatural amino acids using combinatorial methods allows for synthesis of an enormous number of cyclic compounds in libraries of various size. Furthermore, cyclic peptides are conformationally constrained, an asset in molecular modeling studies.

Our initial experiments showed that 7- and 8- residue cyclic peptides with two or three cationic groups had detectable affinity for the P-site at 100 μM concentrations (*5*). The next series of cyclic compounds was modeled on the snake neurotoxin fasciculin, which is specific for the P-site and displays an impressive affinity for the enzyme (K_D ~ 10 - 20 pM) (*6, 7*). Fasciculin consists of three finger-like loops protruding from a central disulfide linked core, and crystal structures of the AChE-fasciculin complex (*8, 9*) show that loop II displays the greatest area of contact with the AChE P-site. The loop II primary sequence -Arg-Arg-His-Pro-Pro-Lys-Met- was cyclized by insertion of a Gln residue, and substitutions at Arg^2 and Lys^6 (which do not make direct contact with the enzyme surface) were investigated. The cyclic peptide cyclo[Arg-Nal-His-Pro-Pro-Lys-Met-Gln], where Nal is napthylalanine, had the highest affinity and was found to inhibit AChE with an inhibition constant K_I of less than 1 μM (*5*). However, we were concerned about too great an emphasis on this fasciculin-based cyclic peptide. The crystal structures showed that insertion of fasciculin loop II into the AChE P-site results in contacts of both faces of loop II with AChE residues. These contacts are extensive, and surface calculations showed that not even a water molecule would be able to enter the active site in the crystal structure of the complex (*9*). Therefore, high affinity cyclic peptides based on fasciculin loop II may be misleading structural prototypes if our goal is to build a high affinity pore at the peripheral site that still allows access of ACh.

If we move beyond fasciculin loop II as a model, it becomes a challenge to identify a cyclic peptide with sufficient affinity for the AChE P-site to serve as a lead compound for further development. To confine candidate peptide interactions to this site, we have developed a procedure for covalently tethering cyclic peptides near the P-site. Tethering is an intermediate strategy, and we anticipate that high affinity inhibitors identified with this approach will retain their high affinity when the tether is removed. The site selected for tethering was H287, a residue on the rim of the P-site but with a side chain that extends outward into solution. We have developed a protocol for tethering prospective compounds to the P-site by preparing a mutant form of the human AChE that substitutes a cysteine for the histidine at residue 287, designated as H287C AChE. Our candidate cyclic peptides include a Lys residue, which we attach to a linker via the ε-amino group of this Lys. The linker terminates with a methanethiosulfonate group (MTS), and reaction of this substituent with the free -SH group of residue C287 forms a covalent disulfide bond that localizes the cyclic peptide to the P-site. After attachment of the cyclic peptide to the enzyme, we can evaluate binding to the enzyme by affinity chromatography on acridinium resin. We have successfully employed this method with a series of tethered cationic ligands that were linked to C287 (*10*). The location of the tethered ligands were predicted by molecular modeling calculations and verified experimentally by testing the modified enzymes for inhibition with propidium (a P-site inhibitor) and tacrine (an A-site inhibitor). We demonstrated that tethered ligands that only reach the P-site selectively blocked propidium inhibition, while tacrine affinity for the A-site decreased when tethered ligands reach the A-site (*10*). The acridinium affinity resin we routinely use for purifying AChEs tightly retains unmodified AChEs, which are released only under stringent conditions when the AChE inhibitor decamethonium is added to the buffer. Tethered ligands that reached the P- or the A-sites interfered with binding of the modified AChE to the acridinium resin and were eluted from the resin by a less stringent salt wash containing 0.5 M NaCl. This observation suggested a selection criterion for the AChEs tethered to cyclic peptides of interest: Those with active sites that, as desired, are strongly blocked by the tethered peptide will elute from acridinium resin under less stringent conditions, while those with unblocked active sites will require decamethonium for elution.

We have recently advanced our strategy of tethering cyclic peptides to the Cys residue of H287C AChE in a number of ways. Improvements have been made to the linker that attaches the cyclic peptide to the enzyme by the incorporation of a miniPEG group which increases solubility and hydrophilicity. Additionally, we have progressed to screening combinatorial sets of cyclic peptide libraries (100/set) in order to speed up the process of lead compound discovery. After selection of compounds with our affinity resin we must identify which of the 100 compounds in the set are responsible for the altered acridinium chromatography affinity profile. We have turned to the use of mass spectrometry (MS) to identify cyclic peptides. After cleaving the tethered ligand with disulfide reduction, we used MALDI-TOF MS to identify the total mass of the released peptide. This is followed by ESI ion trap MS with MS/MS capability to detect specific peptides by their sequence. Candidate peptides will then be subjected to molecular modeling studies to visualize possible conformations with H287C. From these studies we will optimize the lead compound by modifying the side chain to increase specificity, and also alter size of the cyclic peptide pore.

BODY

During the first year of this grant we developed our tethering strategy and introduced a cysteine residue near the rim of the P-site by site-specific mutagenesis to generate recombinant human H287C AChE. Compounds were synthesized with a highly reactive methanethiosulfonyl (MTS) substituent and linked to this cysteine through a disulfide bond. As a proof of concept, we initially used cationic ligands with demonstrated affinity for the AChE A-site that were attached to MTS tethers of various lengths. Some of the enzymes modified with these tethered ligands lost substantial activity, making it difficult to determine their catalytic properties in the presence of residual unmodified AChE. We found that we needed to have an independent measure of AChE concentrations and began labeling the amine groups in H287C AChE by reductive methylation with [^{3}H]formaldehyde and sodium cyanoborohydride (*11*). Additionally, we found that the MTS-derivatized compounds needed to be separated from the unmodified enzyme and began using acridinium affinity chromatography to allow for this separation. Enzymes modified with ligands having a significant effect on the A-site elute in a NaCl wash, while unmodified enzyme elutes only when the AChE inhibitor decamethonium is included in the buffer. We also compared the substrate dependence of hydrolysis rates on the substrate concentration for unmodified H287C as well as 3 modified enzymes and found that 2 of the modified enzymes shifted the hydrolysis curve, with large increases in K_{app} and K_{SS}. Molecular modeling calculations indicated that cationic ligands with tethers of intermediate length bound to the P-site, whereas those with long tethers reached the A-site. These binding locations were confirmed experimentally by measuring competitive inhibition constants K_{I2} for propidium and tacrine, inhibitors specific for the P- and A-sites, respectively. We compared K_{I2} values and found that the shortest tethers had little effect on K_{I2}, giving values similar to unmodified AChE. Medium length tethers interfered with propidium binding to the P-site, while K_{I2} values for tacrine inhibition increased substantially only when ligands had long tethers. These relative changes in propidiuim and tacrine affinities thus provided a sensitive molecular ruler for assigning the binding locations of the tethered cations. Complete details of this work are described in a paper published in 2003 and is appended to this report (*10*).

Cyclic peptide synthesis. We have recently advanced our strategy of tethering cyclic peptides to the Cys residue in H287C AChE in a number of ways. Several synthesis schemes for linker groups that attach the MTS substituent to the cyclic peptide have been compared and evaluated for ease of synthesis, optimum length and solubility. Refinements now include insertion of a hydrophilic group (8-amino-3,6-dioxaoctanoic acid, or "mini-PEG") to increase solubility and hydrophilicity. The ε-amino group of the lysine side chain in the cyclic peptide was acylated with the pentafluorophenyl ester of Fmoc-mini-PEG. After removal of the Fmoc-protecting group, the primary amine in the mini-PEG substituent was acylated with the succinimido-oxyester of MTS-propionic acid to generate the MTS-linked cyclic peptide. (*12*) The resulting linker is about 13 bond lengths. Some 15 new individual MTS-linker-peptides were investigated. We focused on a group of compounds based on the cyclic peptide cyclo-[RAmbLFXQ], where Amb was 3-(aminomethyl)benzoic acid and X was lysine in either the D- or L-configurations (Table 1). These cyclic peptides were investigated because they showed some affinity for the P-site as reversible AChE inhibitors. However, modification of AChE with only 2 of these MTS-linker-peptides (IV and VII) resulted in partial blockade of the AChE active site, as measured by our selection criterion (release of the modified AChE from acridinium resin under less stringent conditions with 0.5 M NaCl).

Confirmation of cyclic peptide tethering by analysis of the kinetics of fasciculin binding. One concern when we fail to see any change in activity or acridinium resin affinity of an AChE after conducting an MTS-linker-peptide modification reaction is whether a significant fraction of the AChE was actually modified. We have developed a kinetic assay that appears to be very sensitive to any modification near residue 287. We have previously demonstrated that fasciculin can be used to monitor interactions at the P-site (*13*). We have now found that we can use changes in the rate constant k_{on} for fasciculin association with AChE to confirm the presence of compounds tethered at the H287C site. In this procedure, acetylthiocholine (AcTCh) hydrolysis is monitored by continuous spectrophotometric assay in 1 ml assay solutions with buffer (20 mM sodium phosphate and 0.02% Triton X-100 at pH 7.0 at 25 °C). Assay solutions include 1 mM AcTCh and 0.33 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and hydrolysis is monitored by formation of the thiolate dianion of DTNB at 412 nm. Association reactions (1 ml) are initiated by adding small volumes of fasciculin (1 nM) to AChE (10 pM), followed by the immediate addition of DTNB and AcTCh. Assay rates v over 2 s intervals are fitted by nonlinear regression analysis to the following equation (eq. 1): $v = v_{final} + (v_{initial} - v_{final})e^{-kt}$, where $v_{initial}$ and v_{final} are the calculated values of v at time zero and at the final steady state when fasciculin binding has reached equilibrium, and k is the observed rate constant for the approach to equilibrium. This k is given by $k = k_{on}$[fasciculin] + k_{off}. Since k_{off} is negligible for fasciculin, k_{on} is obtained simply by dividing k by the fasciculin concentration.

H287C AChE treated with MTS-linker peptide I (Table 1) eluted from acridinium affinity resin only with decamethonium, indicating that any modification was not apparent by this, our primary criterion for P-site blockade. However, the fasciculin binding assay provided a more subtle measure of the tethering of this compound. Essentially the same k values were obtained for wild type AChE and unmodified H287C, but the eluted enzyme treated with compound I, after extensive dialysis to remove decamethonium, displayed a 3- to 4-fold lower k value. (Figure 1.) This result indicated that the attached cyclic peptide did interfere with fasciculin binding. This assay thus can confirm the attachment of linker peptide to H287C AChE even when the peptide fails to alter acridinium resin binding or enzyme activity.

Combinatorial cyclic peptide libraries. The results in the preceding paragraphs in general indicated that we need to increase the diversity of cyclic peptide structures that we are surveying, and we have pursued this goal with combinatorial syntheses and mass spectrometry (MS) identification. We have attached our MTS-linker to 30 combinatorial sublibraries based on the cyclic peptide sequence c(Arg-Xxx-Xxx-Pro-Pro-Lys-Xxx-Asn), where Xxx = D,L-Pro, D,L-Arg, D,L-Tyr, D,L-Leu and D,L-Ser (*14, 15*). This peptide template retains some sequence features of the loop II segment of fasciculin that interacts with the AChE P-site. Each sublibrary has one Xxx fixed, e.g. at position 7, while the other two Xxx remain variable, giving 10 x 10 = 100 compounds in each sublibrary. In an initial trial, we generated a 100-member library based on the cyclic peptide c(Arg-Xxx_1- Xxx_2-Pro-Pro-Lys-Pro-Asn), where position 7 is defined with L-Pro and where Xxx_1 and Xxx_2 are Pro, Arg, Tyr, Leu or Ser in both the D- or L-configurations. A variety of synthetic methods were used to prepare the cyclic compounds (*12, 16*). Typically, a common initial amino acid (Glu, Gln, Asp, or Asn) was attached to a solid phase MBHA resin support, and the derivatized resin was distributed to a synthesis block composed of up to 40 reaction syringes. Defined mixtures of protected amino acids or analogs were coupled in stepwise fashion, and the products were cyclized and cleaved from the resin with liquid anhydrous HF. A linker group was attached to a primary amino group (*e.g.*, the ε-amino group

of lysine) of the cyclic peptides with a terminal MTS substituent. With miniPEG (MTS-CH_2-CH_2-CO-NH-(CH_2-CH_2O)$_2$-CH_2-CO-) as the linker, the ε-amino group of the lysine side chain in the cyclic peptide was acylated with the pentafluorophenyl ester of Fmoc-miniPEG. After removal of the Fmoc- protecting group, the primary amine in the miniPEG substituent was acylated with the succinimido-oxyester of MTS-propionic acid to generate the MTS-linked cyclic peptides. Typical purification by solid phase C18 resin extraction gave 40 – 70% yields, as estimated by analytical HPLC and MALDI-TOF MS.

An MTS-linker peptide mixture corresponding to sublibrary XVI (Table 1) was reacted with H287C AChE, and the modified AChEs were sorted by the selection procedure on acridinium affinity resin. The results provided a strong endorsement of this procedure. About 7% of the modified AChEs failed to bind to the resin and were eluted with the least stringent wash (10 mM sodium phosphate, pH 7), another 20% were eluted in the lower stringency 0.5 M NaCl wash, and the remaining 70% were eluted with decamethonium. (Figure 2). Furthermore, propidium inhibition studies (noted above) revealed striking differences in the extent of P-site blockade in the three fractions (Figure 3). The K_I for propidium increased more than 1000-fold relative to the unmodified AChE for the peak that was eluted with buffer alone, about 15-fold for the second peak eluted with NaCl, and less than 2-fold for the third peak (deca). Reduction of the modified AChEs with dithiothreitol was expected to cleave the disulfide bond between the linker-peptide and residue C287, release the peptide, and restore the propidium affinity. This was also observed. After reduction and dialysis of all three fractions and unmodified H287C AChE, the K_I values for propidium were identical.

Mass spectrometry. Tethered cyclic peptides that strongly block the AChE active site are possible candidates for the lead compound that we seek. Our ability to release these peptides from AChE by disulfide reduction encouraged us to explore the use of MS as a powerful tool for their identification. In 2003 our Department of Neuroscience at Mayo Clinic Jacksonville purchased a ThermoFinnigan DecaXP Plus ESI ion trap mass spectrometer with liquid chromatography interface to support three projects, one of which was this project. This instrument has recently been modified with an Advion NanoMate accessory that allows automated direct injection of small (5 – 10 μl) consecutive samples and thus provides much greater sensitivity. In addition, the department also has an older Applied Biosystems, Voyager-DERP MALDI-TOF mass spectrometer that we have found useful. Bernadette Cusack, a senior research analyst in our laboratory, has spent much of the past two years obtaining training and developing techniques in MS, and we have gained perspective on the most effective ways to apply these techniques to our project. Initial MALDI TOF MS experiments were conducted with H287C AChE modified with a single MTS-linker-peptide, cyclo-[Arg-3-Amb-Leu-Phe-Lys-GlnRAmbLFKQ]. Reduction of this AChE with dithiothreitol released a compound with a mass to charge ratio (*m/z*) of 1039.9 that matched within 1 amu the mass we obtained after reduction of the stock MTS-linker-peptide itself (Figure 4). We also showed that we could obtain sequence information on MTS-linker-peptides with our ESI ion trap mass spectrometer and MS/MS analyses by collision activated decomposition (CAD). For example, fragmentation of one of our linker peptides resulted in cyclic ring opening at three peptide bond sites and release of four 3- to 5-residue fragments (Figure 5). These fragments provided information about the cyclic peptide sequence. We concluded from these studies that we could assign cyclic peptide structures selected from combinatorial peptide mixtures based on the combination of parent ion masses and specific sequence information from MS/MS analyses with CAD.

Initial MS results from the three AChE fractions modified with the 100-member c(Arg-Xxx-Xxx-Pro-Pro-Lys-Pro-Asn) library (Sublibrary XVI in Table 1) and shown in Figure 2 are encouraging. Released peptides could be identified from all three fractions both by MALDI-TOF and by ESI ion trap MS. An application called total ion mapping with the DecaXP Plus ion trap MS identified peptides from the library by focusing on an MS/MS product ion common to all (*e.g.*, *m/z* 556 for the fragment -PK(linker)P-). MS/MS scans were conducted at each amu increment in parent ion spectra between *m/z* 1050-1250, and the ion currents for all parent ions that produced a *m/z* 556 product were graphed. The total ion maps for all three fractions showed 6 – 8 of the 15 possible peptide masses in the library. However, the relative amounts of these peptides were similar and no peptide was uniquely identified in the AChE fraction that showed the greatest P-site blockade. This observation suggested that the MTS-linker-peptides were reacting not only at residue C287 but also at another AChE cysteine residue, probably Cys 551. Cys 551 forms a native intersubunit disulfide bond in our recombinant dimeric human AChE, but it is known to be susceptible to the mild reduction pretreatment that is necessary for MTS modification of H287C AChE. We had hoped to avoid mutating Cys 551 in our recombinant AChE because this disulfide provides some stabilization to the purified enzyme, but we will now engineer the double mutant H287C/C551S AChE for future MTS tethering studies.

Candidate selections from initial library screen. One released peptide detected by MALDI-TOF and total ion mapping MS with a reduced mass *m/z* = 1165.5 was subjected to MS^2 sequencing as shown in Figure 6. This mass corresponds to two possible sequences, c(Arg-D,L-Arg-D,L-Ser-Pro-Pro-Lys-Pro-Asn) (denoted 17-1) and c(Arg-D,L-Ser-D,L-Arg-Pro-Pro-Lys-Pro-Asn) (denoted 18-1), and fragments consistent with both sequences were detected in Figure 6. Another observed released peptide with m/z = 1182.5 corresponded to c(Arg-D,L-Pro-D,L-Tyr-Pro-Pro-Lys-Pro-Asn) (denoted 15-1) and c(Arg-D,L-Tyr-D,L-Pro-Pro-Pro-Lys-Pro-Asn) (denoted 16-1). These sequences all have four stereoisomer alternatives at the second and third amino acid positions, where D- or L- isomers were mixed. To compare these four cyclic peptide sequences, we synthesized each as a mixture of the four stereoisomers and tested them with our labeling and acridinium affinity chromatography selection procedures. After enzyme labeling and affinity chromatography with the two sets 15-1 and 16-1 representing *m/z* = 1182.5, more than 86% of the modified enzyme eluted in the decamethonium peak, indicating that there was minimal inhibition of enzyme activity. However, our results for the two sets 17-1 and 18-1 were more promising (Figure 7). The elution profiles for both sets were very similar. About 9-13% of the modified AChEs failed to bind to the resin and were eluted with the least stringent wash (10 mM sodium phosphate, pH 7). Another 20% were eluted in the lower stringency 0.5 M NaCl wash, and the remaining 57-66% were eluted with decamethonium. These two sets of cyclic peptides also gave very similar results in the fasciculin and propidium competition assays (Table 2.) Results for both sets with the fasciculin assay indicated that the H287C modified enzyme that eluted in the buffer peak (10mM NaPi, pH 7.0) displayed a 2-3-fold lower k_{on} value while the modified enzyme that eluted in the NaCl wash was 5-fold decreased as compared to unmodified enzyme. These results confirm the presence of the cyclic peptides at the P-site. Propidium affinities for these modified AChEs relative to unmodified enzyme were decreased 6-fold for the buffer peak and 6- to 8-fold decreased for the NaCl peaks. (Table 2.) This represents a modest blockade at the P-site. Since both compounds have very similar elution profiles as well as inhibition levels with the fasciculin and propidium assays, the order of Arg and Ser at positions 2 and 3 may not be critical. However, this conclusion is tentative until results with individual stereoisomers are available. We further confirmed the presence of these cyclic

peptides in the modified AChE in the NaCl peak by their release from the enzyme with disulfide reduction and identification by MALDI-TOF analysis. We observed a major peak at m/z = 1167.55. We have begun the synthesis of the stereoisomers individually for both these subsets and will examine them through our protocol of affinity chromatography, fasciculin competition, and propidium inhibition.

Molecular Modeling. We have previously conducted modeling studies of ligand binding to the AChE active site with QUANTA96 software (Molecular Simulations, Inc.) on our Silicon Graphics Indigo2 workstation (*3*). This software has been upgraded to the Affinity docking module in the InsightII package (Accelrys, Inc.) that can use a full molecular mechanics forcefield in searching for and evaluating docked structures. We would like to carry out modeling calculations that would assist cyclic peptide design by predicting relative affinities of tethered peptides to the P-site. We will attempt to extend the modeling calculations obtained with the InsightII package (see Fig. 4 of the appended publication) to tethered peptides that cap the AChE P-site, but this approach has been problematic. The InsightII software, like the majority of both commercially and academically available molecular docking software applications we have explored so far, focuses on elucidating the binding of small, rigid molecules to a cavity removed from aqueous solvent. We require a program that will assist in designing large, flexible tethered cyclic peptides that bind tightly to the P-site of AChE with one face while the other face is left exposed to solvent. While seeking such a program, we are making some progress by adapting our queries to match the capabilities of the software. In InsightII, we have broken up the targeted rim of the P-site into smaller pieces and probed these areas with small, rigid ligands. This approach suggested, for example, that fasciculin-based cyclic peptides were inserting themselves into the P-site rather than binding at the rim as intended.

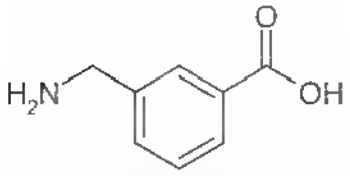
Recombinant AChE expression system. Our peptide identification strategy requires that MS techniques detect and sequence a cyclic peptide released from a modified AChE. Since the amount of peptide is stoichiometrically equivalent to the AChE and may represent only a small percentage of the initial peptide library, success in this MS identification depends on conducting the tethering reaction with a sufficient amount of H287C AChE. We have developed an expression system for recombinant AChE that provides these amounts. This system, which utilizes the Schneider 2 (S2) *Drosophila* cell line and an expression vector with an actin5C promoter, was first used to express stable transfectants of secretion constructs of dimeric *Drosophila* AChE. Up to 50 μg/ml of AChE were accumulated in media after 10-14 days of culture (*17*). We have adapted this system for expression of a soluble dimeric form of recombinant human AChE (*18*). We now produce about 50 mg of human H287C AChE a week by culturing in 8 liters of serum-free media. It should be noted that the maintenance, harvesting, and purification of such large amounts of AChE consumes more than half the effort of a full-time research assistant.

Table 1. MTS-linker-cyclic peptide compounds

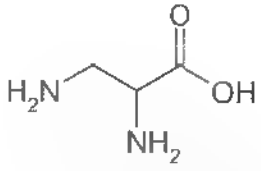
ID	Linker*	Cyclic Peptide
I	MTS-propionyl-mini PEG	c(Arg-3AMB-Leu-Phe-D-Lys-Gln)
II	MTS-propionyl-mini PEG	c(Arg-3AMB-Leu-Phe-Lys-Gln)
III	MTS-propionyl-mini PEG	c(Arg-3AMB-Leu-Phe-DAP-Gln)
IV	MTS-$(CH_2)_2$-CO-	c(Arg-3AMB-Leu-Phe-Lys-Gln)
V	MTS-$(CH_2)_4$-CO-	c(Arg-3AMB-Leu-Phe-Lys-Gln)
VI	MTS-CH_2-C_6H_4-CH_2-CO-	c(Arg-3AMB-Leu-Phe-Lys-Gln)
VII	MTS-$(CH_2)_2$-CO-	c(Arg-3AMB-Leu-Phe-D-Lys-Gln)
VIII	MTS-$(CH_2)_4$-CO-	c(Arg-3AMB-Leu-Phe-D-Lys-Gln)
IX	MTS-CH_2-C_6H_4-CH_2-CO-	c(Arg-3AMB-Leu-Phe-D-Lys-Gln)
X	MTS-propionyl-	c(Arg-3AMB-Leu-Phe-Lys-Gln)
XI	MTS-propionyl-	c(Arg-3AMB-Leu-Phe-DAP-Gln)
XII	MTS-$(CH_2)_4$-CO	Phe-Arg-Lys-Arg-Lys-Arg-Ser-Arg
XIII	MTS-CH_2-C_6H_4-CH_2-CO	Phe-Arg-Lys-Arg-Lys-Arg-Ser-Arg
XIV	MTS-CH_2-C_6H_4-CH_2-CO	Gly-Phe-Arg-Lys-Arg-Lys-Arg-Ser-Arg
XV	MTS-CH_2-C_6H_4-CH_2-CO	c(Arg-Ala-His-Pro-Pro-Lys-[D-Nal]-Asn)
XVI	MTS-propionyl-mini PEG	C(Arg-Xxx-Xxx-Pro-Pro-Lys-Pro-Asn)**

*The linker is attached either to the N-epsilon-amino group of Lys or the N-beta-amino group of diaminopropionic acid.

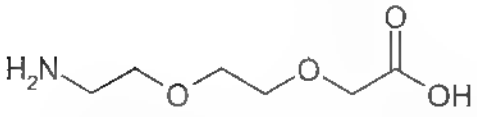
**Xxx – D,L-Pro, D,L-Arg, D,L-Tyr, D,L-Leu, and D, L-Ser.



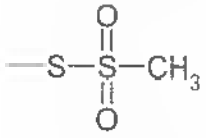
3-(aminomethyl)benzoic acid
(3-AMB)



2,3-diaminopropionic acid
(DAP)



8-amino-3,6-dioxaoctanoic acid
(mini PEG)



Methanethiosulfonyl
(MTS)

Table 2. H287C AChEs labeled with subsets 17-1 and 18-1: Comparison of Fasiculin k rates and propidium K_{I2} after modification.

H287C modification	Fasiculin k, s^{-1}	Propidium K_{I2}, μM
UNMODIFIED	0.5699	0.4120
SUBSET 17-1[+]		
Buffer peak	0.1806	2.3975
Salt peak	0.1180	3.1969
Deca peak	0 3109	0.9677
SUBSET 18-1[++]		
Buffer peak	0.2373	2 3690
Salt peak	0.1143	2.3847
Deca peak	0.3076	0 7171

[+]Subset 17-1 = c(RRSPPK*PN)
[++] Subset 18-1 – c(RSRPPK*PN)
* Denotes location of linker group on Lys residue.

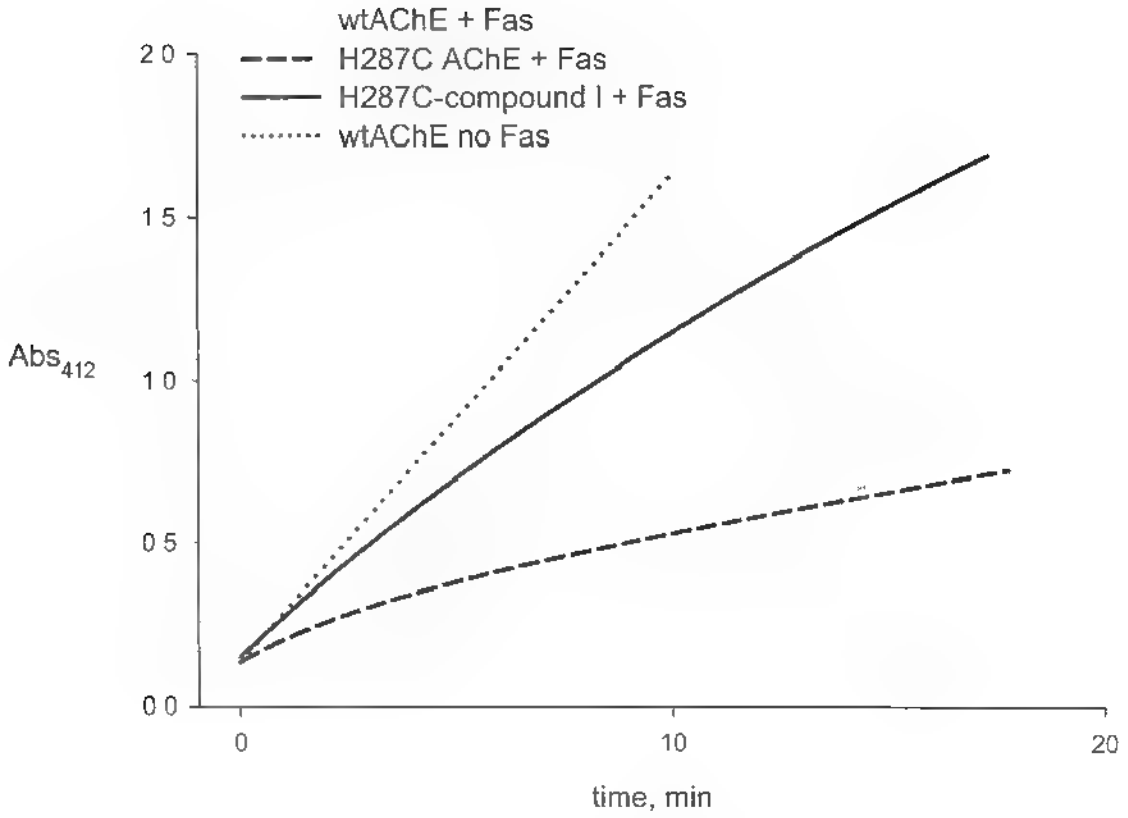


Figure 1. Fasiculin (Fas) association assay with AChE. The rate constant k for fasiculin association was measured according to equation 1 from the rate of approach to the steady state in this continuous spectrophotometric assay of acetylthiocholine hydrolysis. Samples treated with 1 nM Fas at time zero include control wt AChE, H287C AChE, and H287C AChE modified with MTS-linker-peptide I. The measured k values were 0.29 min^{-1} with wt AChE, 0.32 min^{-1} for H287C, and 0.08 min^{-1} for H287C modified with compound I. The dotted line corresponds to an assay with wt AChE in the absence of Fas.

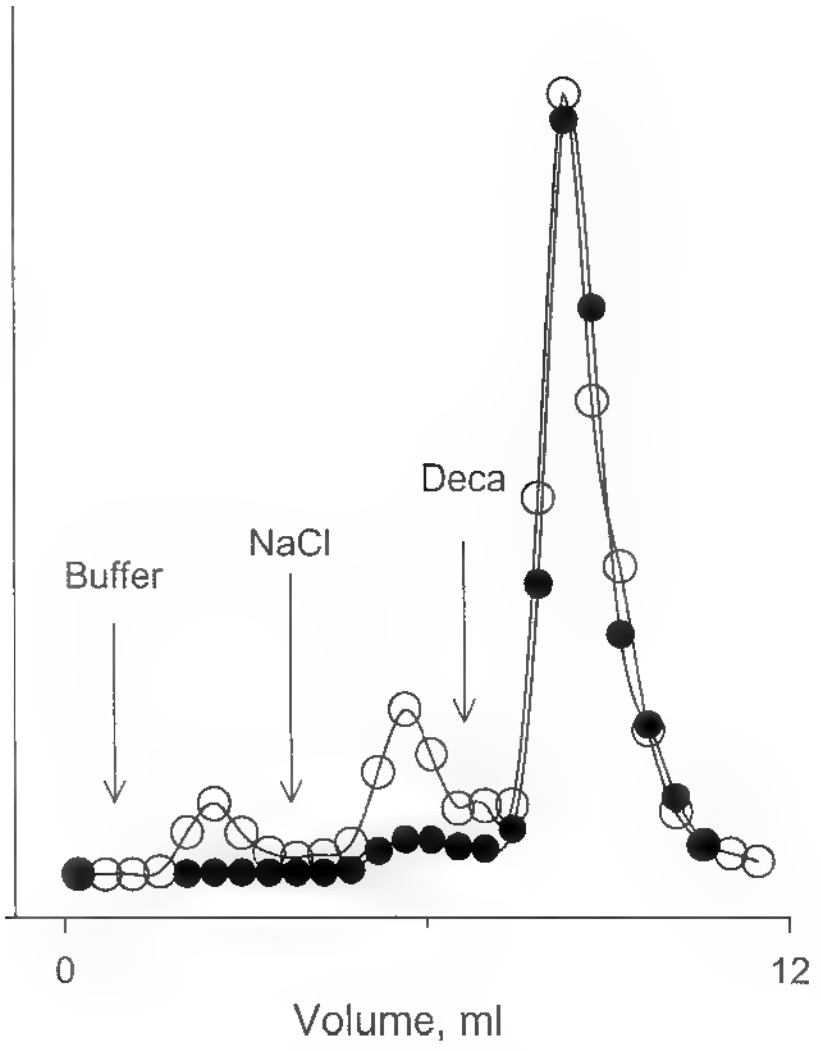


Figure 2. H287C AChE was reductively radiomethylated, treated with an MTS sublibrary XVI (Table 1) and subjected to affinity chromatography on acridinium resin as outlined in the "Body". The resin was washed sequentially with 10 mM sodium phosphate, pH 7.0 (*buffer*), buffer containing 0 5 M NaCl (*salt*), and 5 mM buffer containing 0.5 M NaCl and 5 mM decamethonium bromide (*deca*). Collected fractions were monitored by spectrophotometric assay with 0.5 mM acetylthiocholine for enzyme activity (relative activity, *solid symbols*) and by liquid scintillation counting (relative dpm, *open symbols*). Relative y-axis scales were adjusted to give superposition of the activity and dpm values in the fractions eluted with decamethonium

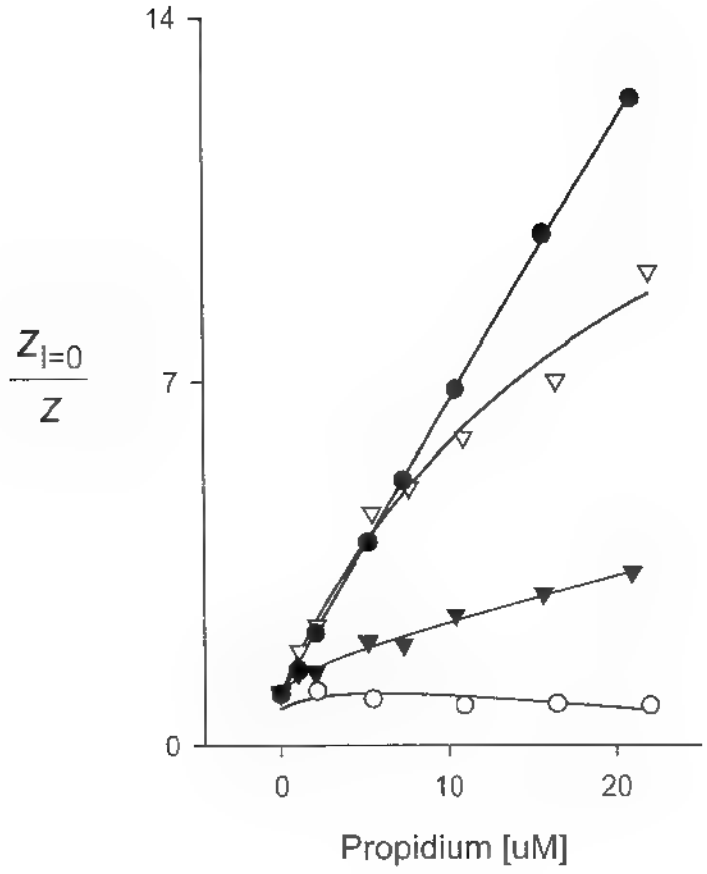


Figure 3. H287C AChE was modified with MTS sublibrary XVI and isolated as shown in Fig 2. Aliquots of these AChEs from the buffer (○), salt (▼) and deca (▽) fractions and unmodified H287C (●) were assayed with 20 μM acetylthiocholine at the indicated propidium concentrations, and relative hydrolysis rate constants ($z_{I\ \ 0}/z$) were determined as outlined before (see Johnson, 2003 for assay details and equations 3 and 4). Data for the unmodified enzyme (●) were fitted with Equation 3 to give K_{I1} = 1.82 μM and a = 0 001 Data for the modified enzyme fractions were fitted with Equation 4 after fixing K_{I1} at 1 82 μM and a at 0.001 and a_2 at 0 001 K_{I2} values obtained from these analyses were as follows: Buffer (○) K_{I2} > 50 μM; salt (▼) K_{I2} = 25.48 μM; deca (▽) K_{I2} = 0.91 μM

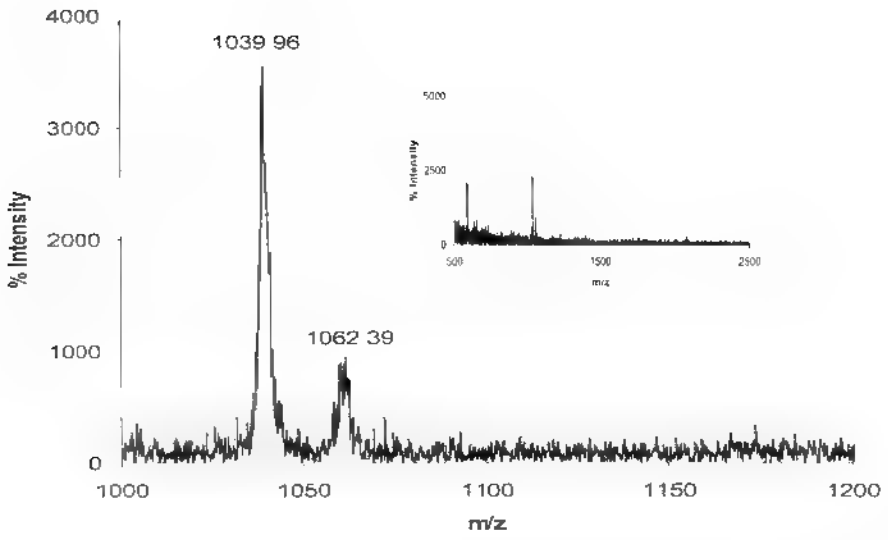


Figure 4. MALDI-TOF scan of reduced H287C modified with compound II (inset) Zoom scan shows a peak with m/z = 1039 96 corresponding to the reduced compound II in Table 1 The minor peak at m/z = 1062.39 represents the sodium adduct of the major peak

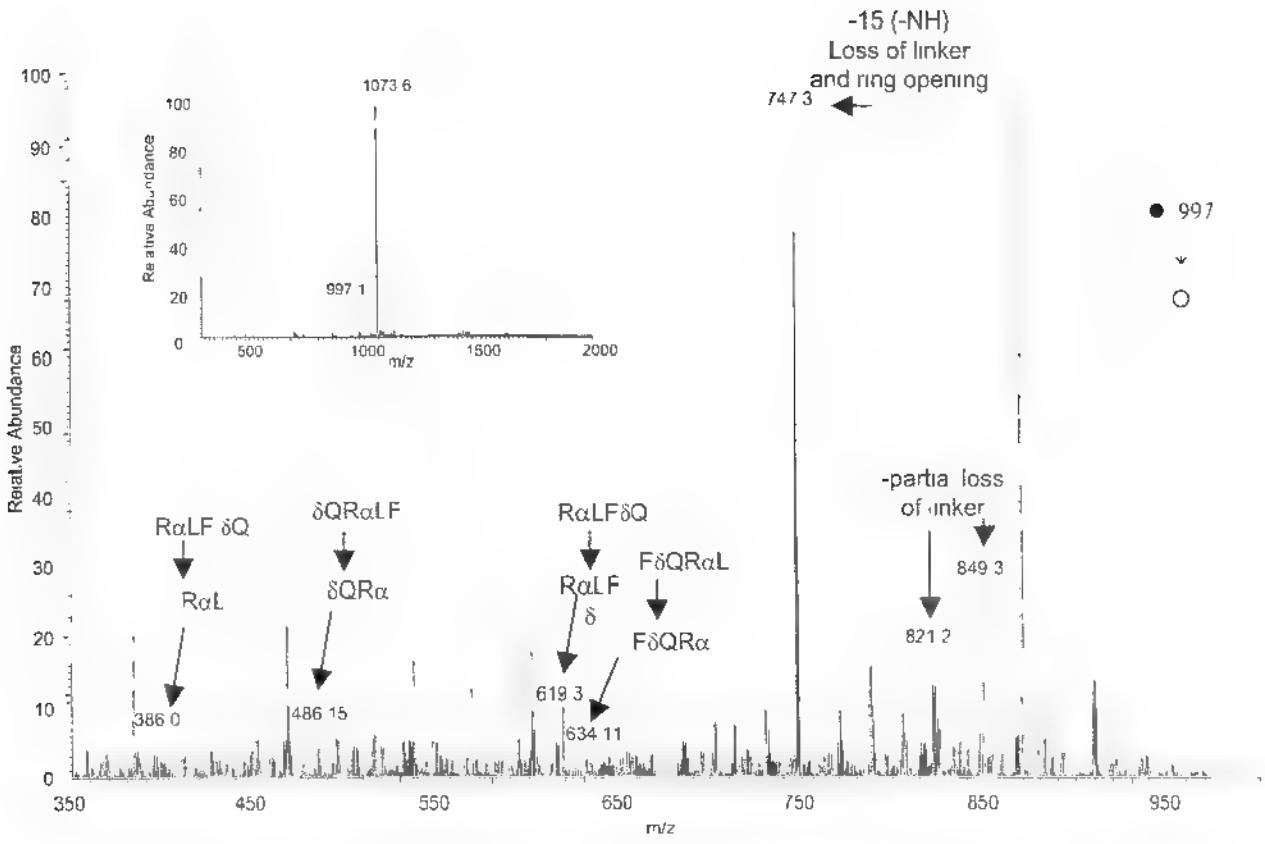


Figure 5. Full MS scan (inset) of MTS-linker-peptide III (MTS-propionyl-mini PEG [c(Arg 3AMB-Leu-Phe-DAP-Gln)] with the ESI ion trap mass spectrometer in direct infusion mode indicates the intact compound (*m/z* = 1073.6) and the reduced compound without the MTS group (*m/z* 998.1). Subsequent MS/MS of the 997 ion generated selected peaks that have been labeled to indicate their significance in the sequence identification of compound III. The prominent peak at 747 3 corresponds to loss of the linker and ring opening with loss of an NH group Additional peaks are labeled with their 6-residue parent sequence, which indicates the peptide bond broken during ring opening, and the subsequent 3- to 5-residue fragment produced (α = 3AMB; δ = DAP).

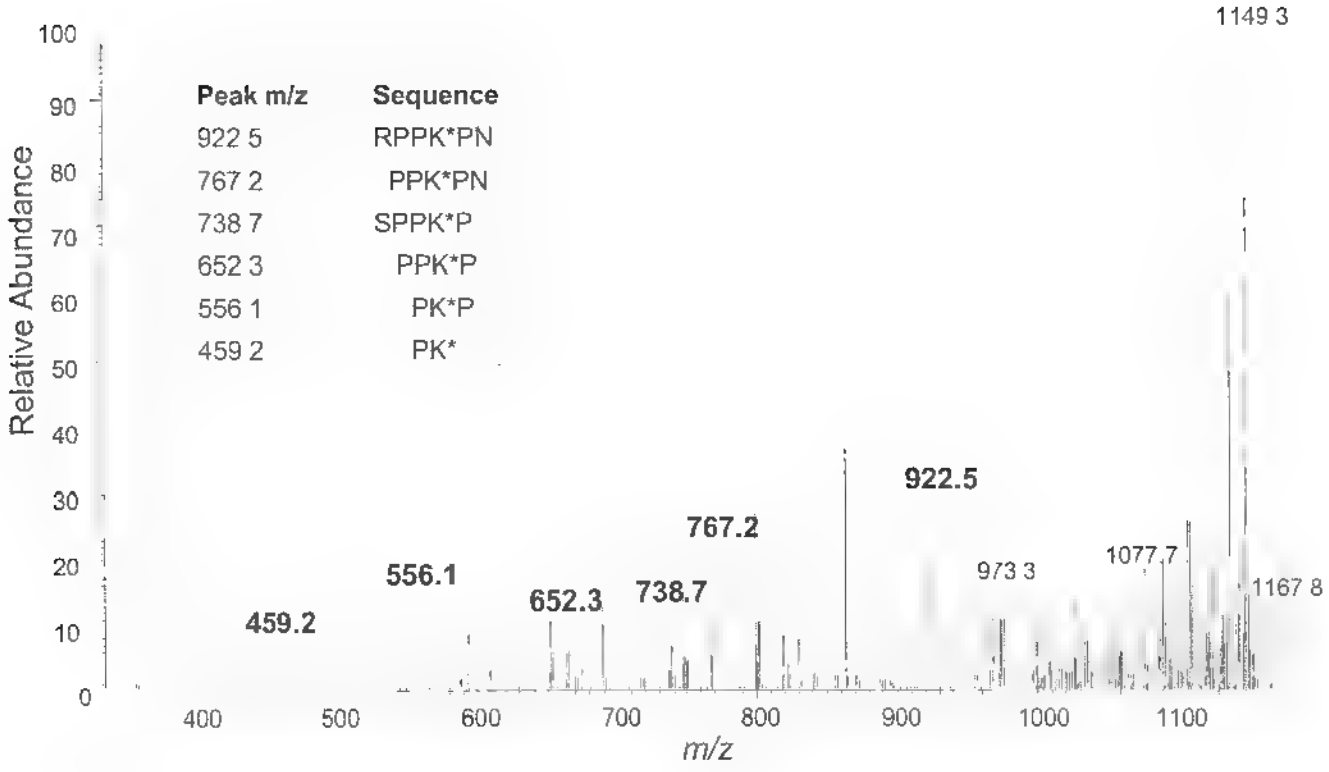


Figure 6. Sequence information from an MS^2 scan of parent ion *m/z* 1165 5. This peptide was released from the fraction of H287C AChE modified with sublibrary XVI that eluted with NaCl in Figure 2. Fractions from the salt peak were dialyzed to remove excess salt, concentrated, and treated with 5 mM dithiothreitol in 10 mM TRIS (pH 8.0) for 1 h at room temperature to release the cyclic peptides. The sample was diluted 1 5 with MeOH·H_2O:Acetic acid (50%:50°₀:0.1%), and a 5-µl aliquot was directly infused with the Nanomate accessory into the ESI ion trap mass spectrometer. An MS^2 scan of the parent ion 1167 *m/z* from the cyclic peptide library identified 6 possible fragment ions that correspond to the reduced *m/z* values for 2 cyclic peptide sequences from our set of 100. The fragment ions and their accompanying sequences are listed in the table. The asterisk indicates the presence of a partial linker on the lysine that is still attached to the peptide fragments. Two sequences are identified for a reduced parent mass of 1167 *m/z*, RRSPPK*PN (17-1) and RSRPPK*PN (18-1)

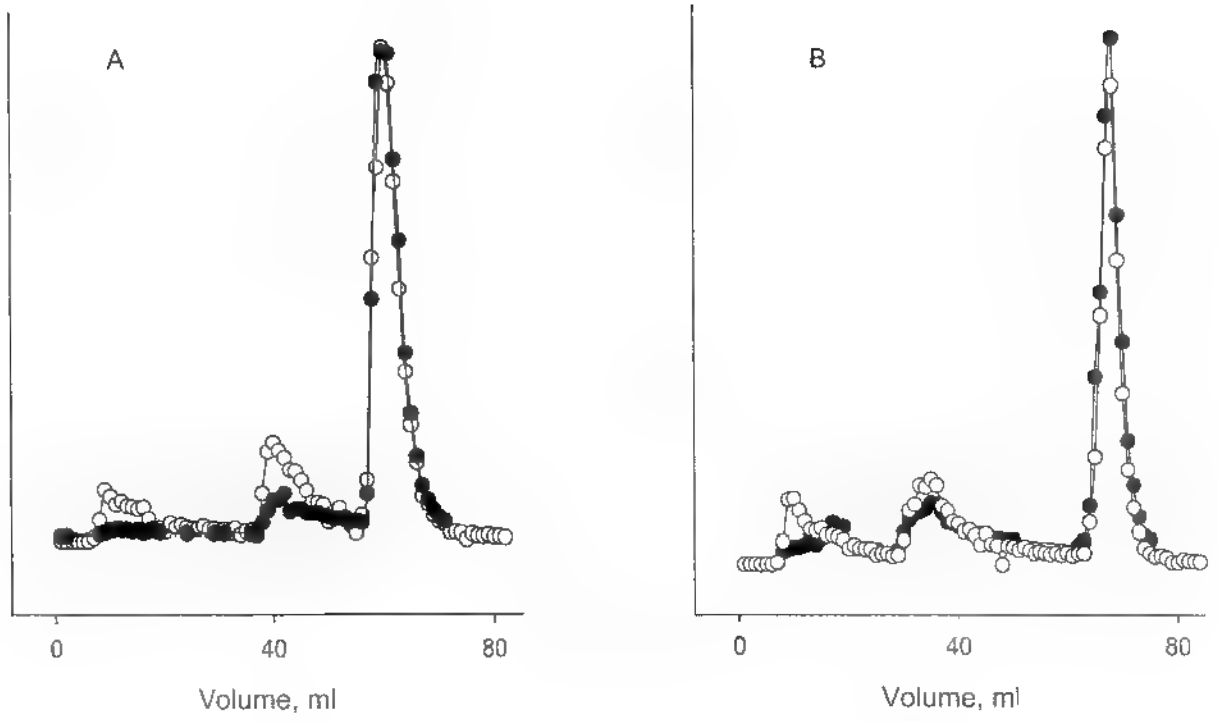


Figure 7. Acridinium resin affinity chromatography of H287C AChEs modified by cyclic peptide subsets MTS-17-1, *panel A*, and MTS-18-1, *panel B*. The resin was washed sequentially as described in figure 2. Collected fractions were monitored by spectrophotometric assay with 0.5 mM acetylthiocholine for enzyme activity (relative activity, *solid symbols*) and by liquid scintillation counting (relative dpm, *open symbols*). Relative y-axis scales were adjusted to give superposition of the activity and dpm values in the fractions eluted with decamethonium.

KEY RESEARCH ACCOMPLISHMENTS

- The development of procedures involving the labeling of H287C enzyme with MTS tethered compounds, have been worked out successfully. These methods include radiomethylation, followed by covalent linkage of MTS tethered compounds to the H287C.

- After labeling of H287C with MTS tethered compounds, we were confronted with the problem of distinguishing the modified enzyme species in which the P-site is at least partially blocked from modified but unblocked species as well as unmodified AChE. Using our knowledge of ligand-based affinity chromatography, we have established a protocol for separating MTS modified enzymes with a partially blocked P-site.

- To facilitate the monitoring of the attachment of MTS-linker-peptides to Cys 287, we developed an assay with the neurotoxin fasciculin, a peptide ligand specific for the P-site. A decrease in the association rate constant k_{on} for fasciculin binding to the P-site indicates attachment of the peptide and at least partial blockade of the P-site.

- We examined substrate dependence and hydrolysis of unmodified H287C and enzyme with cationic trialkyl-ammonium, acridinium, and tacrine with tethers of varying length. Modification by ligands with short tethers had little effect on catalytic properties, but longer tethering resulted in shifts in substrate hydrolysis profiles and reduced affinity for acridinium affinity resin. These kinetic results fit well with the *steric blockade model.*

- Effects of the tethered ligands blocking either the P-site or A-site was compared by testing inhibition with propidium and tacrine By comparing competitive inhibition constants of K_{I2} for propidium and tacrine, inhibitors specific for the P- and A- sites can be determined

- We advanced our novel strategy of tethering MTS cyclic peptides to the H287C mutant AChE to include 15 compounds comparing several linkers. Several synthesis schemes for linker groups that attach the MTS substituent to the cyclic peptide have been compared and evaluated for ease of synthesis, optimum length and solubility. Our results showed that the linker MTS-propionyl-mini-PEG was the best choice.

- We have explored the use of mass spectrometry as a powerful tool to identify candidate cyclic peptides from initial combinatorial sets We demonstrated that we could apply MALDI TOF mass spectrometry to identify the total mass of a linker peptide attached to H287C AChE after releasing it by disulfide reduction.

- Sequence information on MTS-linker-peptides was obtained with our ESI ion trap mass spectrometer and MS/MS analyses by collision activated decomposition (CAD). Fragmentation of the linker peptide corresponding to compound III (Table 1) resulted in cyclic ring opening and release of fragments that provided information about the cyclic peptide sequence.

- The results obtained by modifying H287C with the individual cyclic peptides above in general indicated that we needed to increase the diversity of cyclic peptide structures that we are surveying. We have prepared 30 combinatorial libraries based on the peptide sequence c(Arg-Xxx-Xxx-Pro-Pro-Lys-Xxx-Asn), where Xxx = D,L-Pro, D,L-Arg, D,L-Tyr, D,L-Leu and D,L-Ser. These libraries retain some features of fasciculin loop 2 that interacts with the AChE P-site. Each sublibrary has one Xxx fixed, e.g at position 7, while the other two Xxx remain variable, giving 10 x 10 = 100 compounds in each sublibrary.

- Using one cyclic peptide sublibrary (c(Arg-Xxx-Xxx-Pro Pro-Lys-Pro-Asn)), we have taken a set of 100 compounds through our protocol of labeling H287C, and affinity chromatography separation. The compounds eluting with the salt wash were evaluated with the kinetic assays for fasculin association as well as propidium and tacrine inhibition analyses. To identify the compounds responsible for these kinetic effects, we concentrated the salt peak sample and then released the linker cyclic peptide from the H287C by disulfide reduction. We successfully identified the total mass of the linker peptides using MALDI- TOF mass spectrometry and followed this with sequence identification with ESI ion trap MS and MS/MS.

- After screening this first sublibrary, we selected two candidate cyclic peptide subsets from the set of 100 with reduced m/z = 1165.5 and 1182.5. Each subset corresponds to two possible sequences with four stereoisomeric possibilities. We synthesized each of the four sequences as a mixture of stereoisomers and tested these MTS-linker peptides through our labeling and acridinium affinity chromatography selection procedures. Our results with the cyclic peptides having a reduced mass of 1165.5 (c(Arg-D,L-Arg-D,L-Ser-Pro-Pro-Lys-Pro-Asn) and c(Arg-D,L-Ser-D,L-Arg-Pro-Pro-Lys-Pro-Asn)) indicated that they are providing a modest blockade at the P-site. We have begun synthesis of the stereoisomers individually for both these subsets and will test this next round of cyclic peptides through our selection sequence.

- We have established a stable Schneider 2 Drosophila cell line expressing the H287C mutant AChE. This cell line is producing quantities of this enzyme, about 50 mg a week, to provide us with sufficient protein for the present and proposed studies

REPORTABLE OUTCOMES

BOOK CHAPTERS

Johnson, J L , Cusack, B , Davies, M.P., Fauq, A. and Rosenberry, T.L. Substrate activation with a cationic acetanilide substrate in human acetylcholinesterase. In *Cholinesterases in the Second Millennium: Biomolecular Pathological Aspects.* p. 213-218, N C Inestrosa and E.O. Campos (eds) 2004, Diseno e Impresiones J&J Ltda., Chile

Cusack, B., Johnson, J.L., Hughes, T.F., McCullough, E.H., Fauq, A., Romanovskis, P.V., Spatola, A.F. and Rosenberry, T.L. Tethering of ligands near the active site of acetylcholinesterase mutant H287C: Progress on a new strategy for protection against organophosphate inactivation. In *Cholinesterases in the Second Millennium. Biomolecular Pathological Aspects.* p. 259-264, N.C Inestrosa and E.O. Campos (eds) 2004, Diseno e Impresiones J&J Ltda., Chile

Romanovskis, P., Rosenberry, T.L., Cusack, B. and Spatola, A.F. Methanethiosulfonyl derivatives of linear and cyclic peptides as novel reagents to study topology of active center gorge of acetylcholinesterase. In *Peptide Revolution· Genomics, Proteomics & Therapeutics* p. 359-360 M. Chorev and T.K Sawyer (eds) 2003, American Peptide Society, USA.

PAPERS

Rosenberry, T.L., Johnson, J.L., Cusack, B., Thomas, J.L., Emani, S., Venkatasubban, K.S (2005). Interactions between the peripheral site and the acylation site in acetylcholinesterase. *Chem -Biol. Interact, in press.*

Romanovskis, P., Rosenberry, T.L., Cusack, B. and Spatola, A.F. Methanethiosulfonyl-derivatives of linear and cyclic peptides as novel reagents to study the topology of the active center gorge of acetylcholinesterase. *Biopolymers.* 71:399-399, (2003).

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ABSTRACTS

Romanovskis, P., Cusack, B., Johnson, J L , and Rosenberry, T L Introduction of radiolabel into constructs designed to bind covalently and inhibit acetylcholinesterase. *J. Pept. Sci* 10(S2), 215, 2004

Johnson, J L , Cusack, B., Hughes, T F , McCullough, E H , Fauq, A., Romanovskis, P , Spatola, A.F., and Rosenberry, T.L. Inhibitors tethered near the acetylcholinesterase active site serve as molecular rulers of the peripheral and acylation sites. *American Chemical Society*, September, 2003.

Cusack, B., Johnson, J.L., Hughes, T.F., McCullough, E.H., Fauq, A., Romanovskis, P.V., Spatola, A.F. and Rosenberry, T.L. Tethering of ligands near the active site of acetylcholinesterase mutant H287C: Progress on a new strategy for protection against organophosphate inactivation. *VII International Conference on Cholinesterases.*, November, 2002.

Johnson, J L , Cusack, B., Hughes, T F , McCullough, E.H., Fauq, A , Romanovskis, P , Spatola, A.F. and Rosenberry, T.L. Inhibitors tethered near the acetylcholinesterase active site serve as molecular rulers of the peripheral and acylation sites. *VII International Conference on Cholinesterases* , November, 2002.

Cusack, B , Johnson, J. L., Hughes, T. F., McCullough, E. H., Romanovskis, P. V., Spatola, A. F and Rosenberry, T. L. Modulation of acetylcholinesterase kinetics employing ligands tethered to the mutant H287C: A model for organophosphate inhibitor design. *Bioscience Medical Defense Review*, June, 2002.

Johnson, J. L., Cusack, B., Davies, M. P. and Rosenberry, T. L. Ligand interactions within the active site of acetylcholinesterase. *Bioscience Medical Defense Review*, June, 2002

FUNDING

Interactions in the Active Site of Acetylcholinesterase
PI. Terrone L. Rosenberry
NOT-NS-05-004
NIH

Proposed dates: 6/1/05-5/31/06
Total Costs requested. $76,000
(Supplemental funding)

Assess the role of ligand binding to a peripheral site at the entrance to the AChE active site gorge

EMPLOYMENT

J L. Johnson, Ph.D has accepted a tenure-track position as an assistant professor of biochemistry at the University of Minnesota Duluth, starting in the fall of 2005

PERSONNEL

Terrone L. Rosenberry, Ph.D.	Principal Investigator
Bernadette M. Cusack, M.A.T	Senior Research Analyst
Joseph L. Johnson, Ph.D.	Senior Research Fellow
Peteris J Romanovski, Ph.D.	Senior Research Fellow
Leilani Sonoda	Research Technologist

CONCLUSIONS

Our ultimate goal is to develop cyclic compounds that will effectively block access of toxic organophosphates to the A-site of AChE without restricting access of acetylcholine. The A-site is located at the base of the AChE active site, and we have developed an initial strategy that confines cyclic peptides close to the P-site at the active site entrance. We engineered and expressed the AChE mutant H287C, a mutation near the P-site, and developed a method to synthesize candidate compounds with a methanethiosulfonyl (MTS) group. After reductive methylation of the AChE with [^{3}H]formaldehyde, the MTS tethered compound is linked to residue C287 via a disulfide linkage. Affinity chromatography on acridinium resin is then used to separate modified AChEs with blocked P-sites from those with unblocked sites and from unmodified enzyme. The separation is based on the binding selectivity of AChE to the acridinium resin. That is, unmodified or unblocked enzyme will bind to the column and only elute with the competitive inhibitor, decamethanonium. Enzyme modified with ligands that interfere with the binding of the enzyme to the resin will either not be retained by the resin at all or elute with a mild NaCl wash. As proof of concept we investigated cationic trimethylammonium or acridinium ligands with demonstrated affinity for the AChE active site that were attached to MTS tethers of various lengths. Modification by ligands with short tethers had little effect on catalytic properties, but longer tethering resulted in shifts in substrate hydrolysis profiles and reduced affinity for acridinium affinity resin. Molecular modeling calculations indicated that cationic ligands with tethers of intermediate length bound to the P-site, whereas those with long tethers reached the A-site. These binding locations were confirmed experimentally by measuring competitive inhibition constants K_I for propidium and tacrine, inhibitors specific for the P- and A-sites, respectively. Values of K_I for propidium increased 30 to 100-fold when ligands had either intermediate or long tethers. In contrast, the value of K_I for tacrine increased substantially only when ligands had long tethers (*10*). These relative changes in propidium and tacrine affinities thus provided a sensitive molecular ruler for assigning the binding locations of the tethered cations.

We next synthesized a group of individual compounds based on the cyclic peptide c(Arg-3-AMB-Leu-Phe-Xxx-Gln), where Xxx was either L-Lys, D-Lys or DAP (Table 1). This cyclic peptide was selected because it showed some affinity for the P-site as a reversible AChE inhibitor. Systematic substitutions of three residues in the Xxx position (I-XI in Table 1) and five linker structures were made to investigate the effect of changing the length of the side chain attached to the linker. We applied these tethering and screening procedures to all of the 15 MTS-linker-peptides in Table 1. The results with the individual peptides I-XV in general were disappointing. AChE modification with only two (IV and VII) resulted in any enzyme release from the affinity resin in the NaCl wash, with release in other cases requiring decamethonium. Importantly, we did find that the mini-PEG linker increased solubility and hydrophilicity as well as providing for appropriate linker length. When we did not see any change in activity or acridinium resin affinity of AChEs modified with several of these MTS-linker-cyclic peptides, we were concerned about whether a significant fraction of the AChE was modified. To address this question, we developed a kinetic assay that measures the association rate constant (k_{on}) for the neurotoxin fasciculin. This rate constant is decreased by compounds attached to residue C287. We found that even when a modified AChE failed to show altered acridinium resin

binding or enzyme activity, it gave a lower k_{on} value compared to a control, indicating that a cyclic peptide was attached. (Figure 1).

Since one component of our strategy is to identify the tethered cyclic peptides that are selected by our affinity chromatography screening procedure, we need to characterize those compounds that have blocked access to the P-site Mass spectrometry has emerged as an effective technique for the characterization of peptides and proteins. It has proven to be an indispensable tool because it is able to provide accurate molecular mass for low picomole and even femtomole amounts of peptides. With the addition of MS/MS capabilities, sequence analysis of peptides becomes possible. We explored several MS techniques and developed a protocol for identifying candidate compounds. After reduction and release of the cyclic peptide from the labeled AChE, we successfully identified the total mass of the released compounds using MALDI-TOF analysis Full MS scans on an ESI ion trap followed by MS/MS analysis of the target mass was then used to reveal ring opening of the cyclic peptide and obtain a sequence determination through the identification of the fragment ions (Figure 5).

With our demonstrated ability to label AChE with cyclic peptides and then to release and characterize them, we turned to a combinatorial library to speed the process of finding lead compounds. Having developed our selection and identification techniques with individual cyclic peptides, we focused initially on a 100-member subset of a larger 3000-member cyclic octapeptide library and attached the MTS linker group to the entire set (see Table 1, compound XVI) Our results with this sublibrary were encouraging. After affinity chromatography selection, we pursued the modified AChEs that were eluted in the NaCl peak, our criteria for blockade of the P-site in the modified enzymes Fasciculin competition assays indicated that the k_{on} rate constant was decreased, while propidium and tacrine competition assays also indicated altered affinity to these AChEs, thus satisfying our three criteria for P-site inhibition. MALDI-TOF MS and total ion mapping on the ion trap MS revealed several sets of peptides that were released from this modified AChE pool Released peptides with m/z = 1165.5 corresponding to both possible sequences c(Arg-D,L-Arg-D,L-Ser-Pro Pro Lys Pro Asn) and c(Arg-D,L-Ser D,L-Arg-Pro-Pro-Lys-Pro Asn) were detected by MS MS peptide sequencing on the ion trap (Figure 6). To confirm this result, new mixtures corresponding to MTS-derivatized sets of four stereoisomers c(Arg-D,L-Arg-D,L-Ser-Pro-Pro-Lys-Pro-Asn) (denoted 17-1) and (Arg-D,L-Ser-D,L-Arg-Pro-Pro-Lys Pro-Asn) (denoted 18 1) were synthesized. Modification of H287C AChE with these mixtures again resulted in some modified AChEs in which the P site was blocked (Figure 7 and Table 2). Although both 17-1 and 18-1 demonstrated modest inhibition for the P-site, neither could be considered significantly distinct from the other. We have begun synthesis of individual sequences of both these subsets and will pursue analysis of these with our protocols Our success in identifying and evaluating these compounds from our initial screen of 100 compounds was a strong endorsement of our strategy for screening combinatorial libraries.

As noted above, total ion mapping on the ESI ion trap of peptides released from H287C AChE modified with sublibrary XVI (Table 1) revealed a mixture of peptides derived from the library. This result indicated that we may be labeling not only at residue C287 but also at another AChE cysteine residue, probably C551. This residue is located near the C- terminal end of AChE and is known to be susceptible to mild reduction, a process that we use in our labeling protocol. Therefore, we are now engineering the double mutant H287C C551S AChE for future MTS tethering studies to eliminate the possibility of confounding results.

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Inhibitors Tethered Near the Acetylcholinesterase Active Site Serve as Molecular Rulers of the Peripheral and Acylation Sites*

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The acetylcholinesterase (AChE) active site consists of a narrow gorge with two separate ligand binding sites: an acylation site (or A-site) at the bottom of the gorge where substrate hydrolysis occurs and a peripheral site (or P-site) at the gorge mouth. AChE is inactivated by organophosphates as they pass through the P-site and phosphorylate the catalytic serine in the A-site. One strategy to protect against organophosphate inactivation is to design cyclic ligands that will bind specifically to the P-site and block the passage of organophosphates but not acetylcholine. To accelerate the process of identifying cyclic compounds with high affinity for the AChE P-site, we introduced a cysteine residue near the rim of the P-site by site-specific mutagenesis to generate recombinant human H287C AChE. Compounds were synthesized with a highly reactive methanethiosulfonyl substituent and linked to this cysteine through a disulfide bond. The advantages of this tethering were demonstrated with H287C AChE modified with six compounds, consisting of cationic trialkylammonium, acridinium, and tacrine ligands with tethers of varying length. Modification by ligands with short tethers had little effect on catalytic properties, but longer tethering resulted in shifts in substrate hydrolysis profiles and reduced affinity for acridinium affinity resin. Molecular modeling calculations indicated that cationic ligands with tethers of intermediate length bound to the P-site, whereas those with long tethers reached the A-site. These binding locations were confirmed experimentally by measuring competitive inhibition constants K_{I2} for propidium and tacrine, inhibitors specific for the P- and A-sites, respectively. Values of K_{I2} for propidium increased 30- to 100-fold when ligands had either intermediate or long tethers. In contrast, the value of K_{I2} for tacrine increased substantially only when ligands had long tethers. These relative changes in propidium and tacrine affinities thus provided a sensitive molecular ruler for assigning the binding locations of the tethered cations.

The primary physiological role of acetylcholinesterase (AChE)[1] is to hydrolyze the neurotransmitter acetylcholine at cholinergic synapses The AChE structure has evolved to carry out this hydrolysis at rates that are among the highest known for enzyme-catalyzed reactions (1) One feature of the AChE catalytic pathway is the formation of an intermediate acyl enzyme that is hydrolyzed by water It has long been known that AChE forms an acyl enzyme not only with carboxyl esters like acetylcholine but also with carbamic acid esters and phosphoric acid esters (also called organophosphates or OPs) and that these intermediates differ dramatically in their deacylation rate constants (2–5) In particular, organophosphorylated AChEs are hydrolyzed some 10^{10} times slower than acetylated AChE and are effectively inactivated OP inactivation of AChE results in failure of cholinergic synaptic transmission, deterioration of neuromuscular junctions, flaccid muscle paralysis, and seizures in the central nervous system OPs have been developed not only as pesticides targeted to insect AChEs but also, deplorably, as chemical warfare agents directed at human AChE Therapeutic strategies against OP toxicity have been limited Nearly 50 years ago, Wilson and Ginsburg (6) used the notion of complementarity in the AChE active site to introduce cationic oximes as strong nucleophiles that could reverse AChE organophosphorylation These drugs remain a first line of defense against OP toxicity, but they act only on certain forms of inactivated AChE rather than protecting against OP inactivation itself Important goals of our studies are to obtain new insights into the AChE catalytic mechanism and identify new strategies that may prevent OP inactivation

A number of studies have focused on the structural basis of the high catalytic efficiency of AChE X-ray crystallography (7) revealed a narrow active site gorge some 20 Å deep with two separate ligand binding sites The acylation site (A-site) at the bottom of the gorge contains residues involved in a catalytic triad (His-447, Glu-334, and Ser-203)[2] as well as Trp-86, which orients the trimethylammonium group of acetylcholine prior to hydrolysis The peripheral site (P-site) consists of a binding pocket near the surface of the enzyme at the mouth of the gorge and specifically binds certain ligands like the neurotoxin fasciculin (8–11) and the fluorescent probes propidium (12) and thioflavin T (13) We have recently shown that catalysis is accelerated because cationic substrates transiently bind to the P site en route to the A-site (14, 15). However, ligand binding to the P-site also can inhibit AChE through a process we have

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† We dedicate this manuscript to the memory of co-author Arno F Spatola, who died on July 5, 2003

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[1] The abbreviations used are: AChE, acetylcholinesterase, Boc, *tert* butyloxycarbonyl, BOP, (benzotriazol-1-yloxy)-tris(dimethylamino)-phosphonium hexafluorophosphate; OP, organophosphate MTS, methanethiosulfonyl, HPLC, high-performance liquid chromatography

[2] Throughout this report, residue numbers refer to the human AChE sequence

called *steric blockade* (15, 16). This process involves a decrease in the rate constants with which substrates and their hydrolysis products enter and exit the A-site. The concept of steric blockade has led us to explore a new strategy for the design of drugs to protect AChE from OP inactivation. This strategy is to design cyclic compounds that will bind to the P-site with high affinity and selectively block the access of OPs while allowing entry of acetylcholine.

Several approaches can accelerate the process of identifying cyclic compounds that have high affinity for the AChE P-site. Combinatorial chemistry in conjunction with structure-based design has been successfully employed to design high affinity ligands for therapeutic use. Molecular modeling effectively predicts good lead compounds that can then be refined using combinatorial chemistry approaches. To simplify molecular modeling calculations and to assure that cyclic compounds bind specifically to the rim of the AChE P-site *in vitro*, we have adopted a strategy in which we tether ligands via a disulfide linkage near the P-site. We previously showed that residue His-287 at the rim of the AChE P-site can be covalently linked to platinum(terpyridine) chloride to partially block access to the AChE active site (17). Because wild type human AChE has no free cysteine residues, we have mutated this histidine to a cysteine (H287C) to allow introduction of a wider range of tethered ligands at this location. Prospective compounds are synthesized with an MTS group, a highly reactive functional group that reacts with free cysteines to form a disulfide bond (18), thereby allowing their covalent linkage to H287C AChE. In this report, we test the feasibility of this approach using cationic trialkylammonium, acridinium, and tacrine ligands with demonstrated affinity for the AChE active site that are attached to MTS tethers of various lengths. We show that the length of the tether provides a molecular ruler which determines the catalytic properties of the modified AChEs.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human wild type and H287C mutant AChEs were expressed as secreted dimeric forms in *Drosophila* S2 cells in culture (15, 19) and purified by two cycles of affinity chromatography on acridinium resin (20). The mutant AChE construction and expression methods were similar to that described previously (21). Incorporation of the desired H287C mutation was confirmed by DNA sequencing. MTS reagents **I–III** (Table I) were obtained from Toronto Research Chemicals. Propidium iodide was purchased from Calbiochem and tacrine, from Sigma-Aldrich.

9-[N$^{\gamma}$-(β-MTS-propionyl)-γ-aminopropylamino]acridine (IV) Trifluoroacetate—Compound **IV** was prepared in 81% yield by reacting 9-γ-aminopropylaminoacridine dihydrobromide (22) at room temperature with β-MTS-propionic acid (2-carboxyethyl methanethiosulfonate) (Toronto Research Chemicals, Inc.) (1.2 eq) and diisopropylethylamine (4 eq) in the presence of activating agent BOP (1.3 eq) in *N,N*-dimethylformamide. The resulting compound **IV** was purified by reverse-phase HPLC on a Vydac C-18 column (2.2 × 25 cm). ^{1}H NMR (300 MHz, CDCl3) δ 9.71 (br t, NH), 8.11 (d, 2H, 7.4 Hz), 7.91 (br t, NH), 7.65 (br d, 2H, J = 7.2 Hz), 7.47 (t, 2H, J = 7.2 Hz), 7.16 (t, 2H, J = 7.4 Hz), 4.01 (br m, 2H), 3.53 (br m, 2H), 3.47 (t, 2H, J = 6.1 Hz), 3.36 (s, 3H), 2.87 (t, J = 6.6 Hz), 2.14 (m, 2H); (M_r calc = 418; MS (FAB) [M + H]$^+$ = C. O. 418).

9-[N$^{\gamma}$-(N$^{\epsilon}$-β-MTS-propionyl-ε-aminocaproyl)-γ-aminopropylamino]acridine (V) Trifluoroacetate—Compound **V** was prepared in 40% yield by reacting 9-[N$^{\gamma}$-(ε-aminocaproyl)-γ-aminopropylamino]acridine dihydrobromide (22) at room temperature with β-MTS-propionic acid (2.0 eq) in the presence of diisopropylethylamine (4 eq) and BOP (1.3 eq) in dimethylformamide. The resulting compound **V**, after work-up, was purified by HPLC as described for compound **IV**. ^{1}H NMR (300 MHz, CDCl3) δ 9.77 (br t, NH), 8.17 (d, 2H, 8.9 Hz), 7.73 (d, 2H, J = 8.7 Hz), 7.47 (t, 2H, J = 8.7 Hz), 7.26 (t, 2H, J = 8.9 Hz), 6.69 (br t, NH), 4.01 (br m, 2H), 3.51 (m, 2H), 3.42 (t, 2H, J = 6.6 Hz), 3.34 (s, 3H), 3.20 (m, 2H), 2.71 (t, 2H, J = 6.6 Hz), 2.27 (t, 2H, J = 6.9 Hz), 2.13 (m, 2H), 1.64 (m, 2H), 1.52 (m, 2H), 1.35 (m, 2H); (M_r calc = 530; MS (FAB) [M + H]$^+$ = 531).

9-[(4-MTS-methylphenylacetyl)glycyl-7′-amidoheptyl]amino-1,2,3,4-tetrahydroacridine (VI)—4-MTS-methylphenylacetic acid (**VII**) was synthesized in 40% yield from sodium methanethiosulfonate (23) and 4-bromomethylphenylacetic acid following a general method (24); m.p. 130–131 °C (M_r calc = 260; found [M + Na]$^+$ = 283; [M + K]$^+$ = 299). The *N*-hydroxysuccinimidyl ester (**VIII**) of VII was prepared in 80% yield by dropwise addition of dicyclohexylcarbodiimide (1.1 eq) to VII and *N*-hydroxysuccinimide (1.1 eq) in acetonitrile; m.p. 129–131 °C.

9-Chloro-1,2,3,4-tetrahydroacridine (**IX**) was obtained from 1,2,3,4-tetrahydro-9-acridanone and $POCl_3$ (10 eq) after boiling under reflux for 5 h (yield, 85%; m.p. 65–67 °C (lit. (25) 67–68 °C)). Reaction of **IX** with 1,7-diaminoheptane (3 eq) in 1-pentanol (25) gave 9-(7′-aminoheptyl)amino-1,2,3,4-tetrahydroacridine (**X**), which was successfully separated from the disubstituted product and isolated after reverse phase HPLC purification as an oil (M_r calc = 311; [M + H]$^+$ = 312). Reaction of X with the *N*-hydroxysuccinimidyl ester of Boc-glycine in Me_2SO (1 eq) provided 9-(Boc-glycyl-7′-amidoheptyl)amino-1,2,3,4-tetrahydroacridine (**XI**) as an oil (M_r calc = 468; found [M + H]$^+$ = 469). The Boc-group was cleaved with 50% trifluoroacetic acid in dichloromethane, and purification provided 9-(glycyl-7′-amidoheptyl)amino-1,2,3,4-tetrahydroacridine (**XII**) as a light oil (M_r calc = 368; found [M + H]$^+$ = 369). Condensation of XII with VIII (1 eq) provided the desired compound **VI**, which was isolated after reverse phase HPLC purification and freeze-drying. Purity of the product by analytical reverse-phase HPLC was >76% (M_r calc = 610; found [M + H]$^+$ = 611).

Reaction of MTS Reagents I–VI with Radiomethylated H287C AChE—Preparations of H287C were reductively radiomethylated with [^{3}H]HCHO and sodium cyanoborohydride to allow better quantification of AChE protein following MTS labeling (26). This procedure converts the primary amino groups at the N terminus and seven lysine residues in each AChE subunit to di-[^{3}H]methylamines but has no effect on enzyme activity. Dialyzed H287C AChE was pretreated with 1 mM dithiothreitol in 20 mM sodium phosphate (pH 8.0) for 1 h at room temperature to ensure that the cysteine-free sulfhydryl group had not partially oxidized (27), and excess dithiothreitol was removed by dialysis against 10 mM buffer (sodium phosphate, pH 7.0) in a Slide-a-Lyzer (Pierce) with 10,000 M_r cutoff. MTS reagents corresponding to **I–V** were dissolved at 80–120 mM in 50 mM acetic acid. **VI** was dissolved at 1.8 mM because of lower solubility. The concentrations of **IV–VI** were established by absorbance (acridinium, ϵ_{410} = 8.08 mM^{-1} cm^{-1}; tacrine, ϵ_{323} = 11.6 mM^{-1} cm^{-1}). The compounds were then diluted to 6 mM (190 μM for **VI**) with AChE (3–20 μM) in 10 mM buffer and incubated for 30 min at room temperature. The AChE sulfhydryl group displaced methanesulfinic acid from the reagents to form a disulfide bond, and the modified enzymes were dialyzed against 10 mM buffer and applied to an acridinium resin (20) affinity column (1–5 ml). The column was washed sequentially with 10 mM buffer, 10 mM buffer containing 0.5 M NaCl, and 5 mM buffer containing 0.5 M NaCl and 5 mM decamethonium bromide. Collected fractions were monitored for enzyme activity with the Ellman assay and for protein content by liquid scintillation counting.

Steady-state Measurements of AChE-catalyzed Substrate Hydrolysis—Hydrolysis of acetylthiocholine was monitored with a spectrophotometric Ellman assay (28). Assay solutions included 0.33 mM 5,5′-dithiobis(2-nitrobenzoic acid), and hydrolysis was monitored by formation of the thiolate dianion 3-carboxy-4-nitrothiophenol at 412 nm ($\Delta\epsilon_{412\ nm}$ = 14.15 mM^{-1} cm^{-1} (29)). Hydrolysis rates v were measured at various substrate concentrations [S] in 20 mM sodium phosphate, 0.02% Triton X-100 (pH 7.0) at 25 °C, and constant ionic strength was maintained with 0–60 mM NaCl (14). The dependence of v on [S] for unmodified AChE was fitted to Equation 1, the Haldane equation for substrate inhibition (30), by weighted nonlinear regression analysis (assuming constant percent error in v) with Fig.P (BioSoft, version 6.0).

$$v = \frac{V_{max}[S]}{[S]\left(1 + \frac{[S]}{K_{SS}}\right) + K_{app}} \qquad \text{(Eq. 1)}$$

In Equation 1, $V_{max} = k_{cat}[E]_{tot}$, where k_{cat} is the maximal substrate turnover rate, $[E]_{tot}$ is the total concentration of AChE active sites, K_{SS} is the substrate inhibition constant, and K_{app} is the apparent Michaelis constant. $[E]_{tot}$ values for both wild type and unmodified H287C AChE were calculated by assuming 450 units/nmol (13, 15),[3] and for radio-

[3] One unit of AChE activity corresponds to 1 μmol of acetylthiocholine hydrolyzed/min under standard pH-stat assay conditions at pH 8 (13, 20). Our conventional spectrophotometric assay at 412 nm is conducted in pH 7 buffer. With wild type AChE and 0.5 mM acetylthiocholine, this assay results in 4.8 $\Delta A_{412\ nm}$/min with 1 nM AChE or about 76% of the pH-stat assay standard.

methylated AChE these $[E]_{tot}$ values were converted to dpm/nmol ratios that were also applied to the modified AChEs.

Some residual unmodified H287C AChE remained after treatment with the MTS reagents, and fractionation of the reaction mixtures by affinity chromatography did not completely resolve the two enzyme populations. Because substrate hydrolysis by these two AChEs was characterized by different kinetic parameters, Equation 1 was extended to Equation 2.

$$v = \frac{V_{max1}[S]}{[S]\left(1 + \frac{[S]}{K_{SS}}\right) + K_{app}} + \frac{V_{max2}[S]}{[S]\left(1 + \frac{[S]}{K_{SS2}}\right) + K_{app2}} \quad \text{(Eq. 2)}$$

Hydrolysis rates in Equation 2 are the sum of two terms from the Haldane equation. $V_{max1} = k_{cat}[E]_1$ and $V_{max2} = k_{cat2}[E]_2$. V_{max1}, $[E]_1$, k_{cat}, K_{SS}, and K_{app} refer to the unmodified AChE population and V_{max2}, $[E]_2$, k_{cat2}, K_{SS2}, and K_{app2}, to the modified. When fitting data for these AChE mixtures, k_{cat}, K_{SS}, and K_{app} were fixed at the values obtained for unmodified H287C AChE; $[E]_{tot}$ was fixed at values calculated from dpm/nmol ratios; $[E]_1$ was set to $[E]_{tot} - [E]_2$; and k_{cat2}, $[E]_2$, K_{SS2}, and K_{app2} were allowed to vary.

Inhibition Constants for Reversible Inhibitors—At low concentrations of substrate S with a homogeneous AChE, hydrolysis rates are determined by the second order hydrolysis rate constants z. In the absence of inhibitor, z is denoted $z_{I=0}$ and corresponds to V_{max}/K_{app}. Measured z at various fixed [I] were fitted according to Equation 3 by weighted nonlinear regression analyses to obtain the inhibition constant K_I and the experimental parameter α (13, 16).

$$\frac{v_{I=0}}{v} \approx \frac{z_{I=0}}{z} = \frac{\left(1 + \frac{[I]}{K_I}\right)}{\left(1 + \frac{\alpha[I]}{K_I}\right)} \quad \text{(Eq. 3)}$$

In Equation 3, values of z and $z_{I=0}$ either were determined as pseudo first-order rate constants ($[S] = [S]_{initial}e^{-zt}$ (14)) or approximated by single rate measurements v and $v_{I=0}$, in all cases at low substrate concentration ($[S] < 0.2K_{app}$). K_I is the equilibrium dissociation constant for I with E, and the constant α is the ratio of the second order rate constant with saturating I to that in the absence of I. With acetylthiocholine, $0 < \alpha < 0.1$ for P-site inhibitors (16) and $\alpha \cong 0$ for A-site inhibitors.

With two distinct populations of AChE composed of H287C AChE partially modified by MTS reagents, Equation 3 may be extended to Equation 4.

$$\frac{v_{I=0}}{v} \approx \frac{z_{I=0}}{z} = B\left[\frac{(1 - R_2)\left(1 + \frac{\alpha[I]}{K_I}\right)}{\left(1 + \frac{[I]}{K_I}\right)} + \frac{R_2\left(1 + \frac{\alpha_2[I]}{K_{I2}}\right)}{\left(1 + \frac{[I]}{K_{I2}}\right)}\right]^{-1} \quad \text{(Eq. 4)}$$

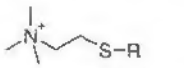
In Equation 4, K_I and α refer to the unmodified AChE population, K_{I2} and α_2 refer to the modified enzyme, and R_2 is the fraction of total activity contributed by the second population in the absence of inhibitor. Data were fitted to Equation 4 by weighted nonlinear regression analysis with K_I and α fixed at the values obtained for unmodified H287C AChE and α_2 assigned equal to α. The B term reduced the emphasis on the $v_{I=0}$ point and was 0.99 ± 0.06 for all analyses.

Molecular Modeling—Molecular modeling was performed with InsightII software (Accelrys, Inc.) on a Silicon Graphics Indigo II workstation. The crystal structure of *Torpedo californica* AChE complexed with tacrine (1ACJ) was taken as the initial structure, and incompletely resolved residues were fully defined using InsightII. Hydrogens were added at pH 7.0, and the resulting structure was energy-minimized for several days to eliminate steric overlap. The histidine at position 287 was mutated *in silico* to a cysteine, and reagents in Table I were attached to H287C through a disulfide linkage. Energy minimizations were initiated from three different placements of the tethered ligand: extending away from the enzyme, into the P-site, and into the A-site. For tethers too short to reach the A-site, the carbon-carbon bonds were stretched until the quaternary amine group was within contact distance of Trp-86. Five computation cycles were performed, each involving energy minimization with the Discover 3 force fields in InsightII followed by a 250-fs interval of molecular dynamics at elevated temperatures (1000–2000 K). This prevented entrapment of the structure in a local energy minimum. The total potential energy function contained a van der Waals contact term (which included hydrophobic and aromatic interaction energies) and an electrostatic term. The peptide backbone and AChE side chains were fixed during energy minimizations with two exceptions: For minimizations in which tacrine or acridinium had to traverse a constriction between the P-site and the A-site (**V** and **VI** starting outside the A-site and **IV** starting in the A-site), Phe-337 and Tyr-72 were allowed to move. Without this flexibility the ligands were trapped and could not traverse the energy barrier at the constriction. Nevertheless, by the final cycle of energy minimization, Phe-337 and Tyr-72 had returned to their starting positions. When **III–V** were initially placed outside the active site, local energy minima were reached with the quaternary amine equidistant from Asp-74 and Asp-292. This location was an artifact, apparently resulting from the absence of explicit waters in the minimizations and the inappropriate extension of electrostatic interactions through the vacuum. To obtain the structures in Fig. 4 and the corresponding potential energy values in Table IV, Asp-292 was mutated to alanine to remove these local minima and allow significantly lower minima to be attained with the quaternary amine close to Asp-74.

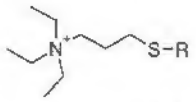
TABLE I

Tethered ligands introduced in H287C AChE by MTS reagents

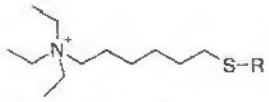
Structures **I–VI** refer both to the MTS reagents, where R = $-S-(O)_2CH_3$, and to the tethered ligands following modification of H287C, where R = $-S-X$ and X is the C_β atom of residue 287 in H287C AChE.



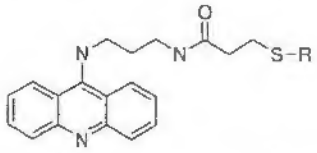
I MTS-ethyl-2-(trimethylammonium)



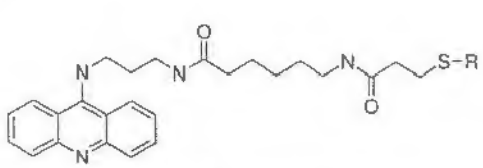
II MTS-propyl-3-(triethylammonium)



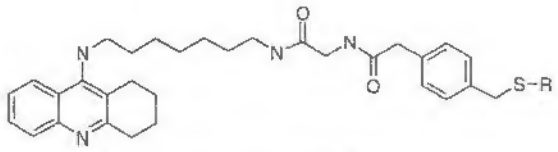
III MTS-hexyl-6-(triethylammonium)



IV 9-[N$^{\gamma}$-(β-MTS-propionyl)-γ-aminopropylamino]acridine



V 9-[N$^{\gamma}$-(N$^{\varepsilon}$-β-MTS-propionyl-ε-aminocaproyl)-γ-aminopropylamino]acridine



VI 9-[(4- MTS-methyl-phenylacetyl)-glycyl-7'-amidoheptyl]-amino-1,2,3,4-tetrahydroacridine

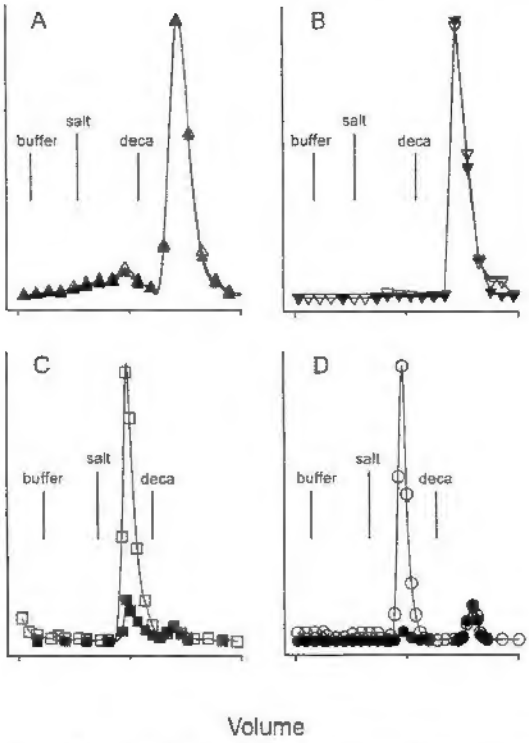


FIG. 1. **Acridinium resin affinity chromatography of H287C AChEs modified by MTS reagents.** H287C AChE was reductively radiomethylated, treated with MTS reagents **II** (*panel B*, ▽, ▼), **IV** (*panel C*, □, ■), or **V** (*panel D*, ○, ●) or left untreated (*panel A*, △, ▲) and subjected to affinity chromatography on acridinium resin as outlined under "Experimental Procedures." The resin was washed sequentially with 10 mM sodium phosphate, pH 7.0 (*buffer*), buffer containing 0.5 M NaCl (*salt*), and 5 mM buffer containing 0.5 M NaCl and 5 mM decamethonium bromide (*deca*). Collected fractions were monitored by spectrophotometric assay with 0.5 mM acetylthiocholine for enzyme activity (relative activity, *solid symbols*) and by liquid scintillation counting (relative dpm, *open symbols*). Relative *y*-axis scales were adjusted to give superposition of the activity and dpm values in the fractions eluted with decamethonium.

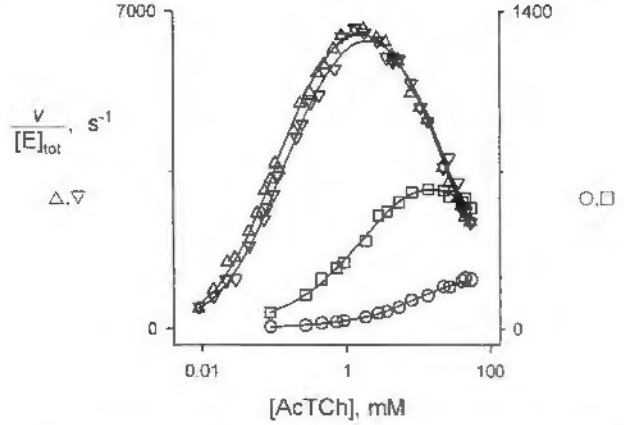


FIG. 2. **Dependence of normalized hydrolysis rates on the acetylthiocholine concentration for H287C AChEs modified by MTS reagents.** Hydrolysis rates v were measured at the indicated acetylthiocholine concentrations as outlined under "Experimental Procedures." Values were obtained for unmodified H287C AChE (△) and H287C AChEs modified with MTS reagents **II** (▽), **IV** (□), and **V** (○) and isolated as shown in Fig. 1. The line for unmodified AChE was calculated by fitting the data to Equation 1. Lines for the modified AChEs were calculated by fitting the data to Equation 2. Each data set was normalized by dividing the measured activity by the concentration of total enzyme present in the assay: unmodified H287C AChE (△), 87 pM; **II** (▽), 61 pM; **IV** (□), 510 pM; and **V** (○), 980 pM. The calculated percentages of modified enzyme in the preparations were 31 ± 20, 98 ± 1, and 99 ± 1, for **II**, **IV**, and **V**, respectively.

RESULTS

Radiomethylation of H287C AChE and Modification with MTS Reagents—H287C AChE was covalently modified with the MTS reagents indicated by **I–VI** in Table I. The reagents lost methanesulfinic acid as they became tethered through a disulfide bond to the sulfhydryl group at residue Cys-287. Preliminary experiments indicated that tethered **I–III** with relatively short cationic groups had minimal effects on enzyme catalytic activity, whereas the larger cationic groups in **IV–VI** gave a pronounced reduction of activity. In fact, these larger groups decreased the catalytic activity to such an extent that residual unmodified AChE obscured the catalytic properties of the modified AChEs. Two procedures were implemented to circumvent this problem. First, stock H287C AChE was reductively radiomethylated to provide a sensitive radioassay of the AChE concentration in subsequent modification and purification steps. In addition, small acridinium affinity columns were introduced to separate H287C AChE treated with MTS reagents into three fractions (Fig. 1). The small amount of AChE inactivated by the labeling procedures was not retained by the column at all and was eluted in the initial buffer wash. H287C AChEs modified with larger cationic groups were bound to the resin with relatively low affinity. Merely increasing the ionic strength of the buffer solution to 0.5 M NaCl released these modified enzymes from the acridinium resin. AChEs modified with **IV**, **V**, and **VI** were in this category (Fig. 1, *C* and *D*). Finally, unmodified AChE and AChEs modified with the smaller cationic groups in **I**, **II**, and **III** bound with high affinity to the resin and were only eluted with high concentrations of the competing AChE inhibitor decamethonium (Fig. 1, *A* and *B*).

Examination of the affinity chromatography profiles revealed that the relative catalytic activity per enzyme site (enzyme activity/dpm) was much lower for the modified AChEs eluted with salt than for the residual unmodified enzyme eluted with decamethonium (Fig. 1, *C* and *D*). To confirm such a change in relative activity, we next compared the dependence of hydrolysis rates v on the acetylthiocholine concentration for the enzymes purified in Fig. 1. Unmodified H287C AChE, like the wild type enzyme, gave a profile in which the activity proceeded through a maximum at intermediate substrate concentrations and showed substrate inhibition at high concentrations (Fig. 2). Values for the Michaelis constant K_{app} and the substrate inhibition constant K_{SS} obtained from Equation 1 were typical of wild type AChE (Table II). Equation 2 was introduced to compensate for residual unmodified AChE in calculating these kinetic parameters for modified AChE preparations. This compensation made little difference for H287C AChE modified with **II**, because similar K_{app} and K_{SS} values were obtained with Equations 1 or 2 (Table II). This result indicates that the tether in **II** is too short to allow the quaternary amine to interfere with substrate hydrolysis. In contrast, the hydrolysis profiles for H287C AChE modified with **IV** and **V** were shifted to the right in Fig. 2, and no substrate inhibition was apparent following modification with **V**. Analysis of these profiles with Equation 2 confirmed that these modifications resulted in large increases in the values of K_{app2} and K_{SS2} and a large decrease in k_{cat2} (Table II). K_{app2} for H287C modified with **V** was shifted to a value nearly 100-fold higher than that for the unmodified enzyme. Furthermore, the relative activity per enzyme site following modification with **V** was significantly lower than that for unmodified H287C AChE (400-fold lower at 0.1 mM acetylthiocholine). These observations indicated that modification with **V** strongly blocked access of acetylthiocholine to the acylation site. Modification with **IV** resulted in

TABLE II
Kinetic parameters for acetylthiocholine hydrolysis with H287C AChEs modified by MTS reagents

H287C modification	K_{SS}[a]	K_{app}[a]	k_{cat}[a]	K_{SS2}[a]	K_{app2}[a]	k_{cat2}[a]
	mM		s^{-1}	*mM*		s^{-1}
Wild-type AChE	22 ± 1[b]	0.076 ± 0.002[b]	6600 ± 300			
Unmodified	23 ± 1	0.11 ± 0.01	7400 ± 400			
II	26 ± 1	0.14 ± 0.01	7200 ± 600			
II	23[c]	0.11[c]	7400[c]	31 ± 7	0.27 ± 0.22	7300 ± 700
IV	23[c]	0.11[c]	7400[c]	200 ± 50	2.0 ± 0.2	680 ± 20
V	23[c]	0.11[c]	7400[c]	—[d]	9.5 ± 1.0	250 ± 10

[a] Data were fitted to Equations 1 or 2 as shown in Fig. 2.
[b] From Ref. 16.
[c] K_{SS}, K_{app}, and k_{cat} were fixed to the fitted values for unmodified H287C. Data for H287C modified with **II** were analyzed with both Equations 1 and 2 as shown.
[d] The fitted $K_{SS2}^{-1} \approx 0$.

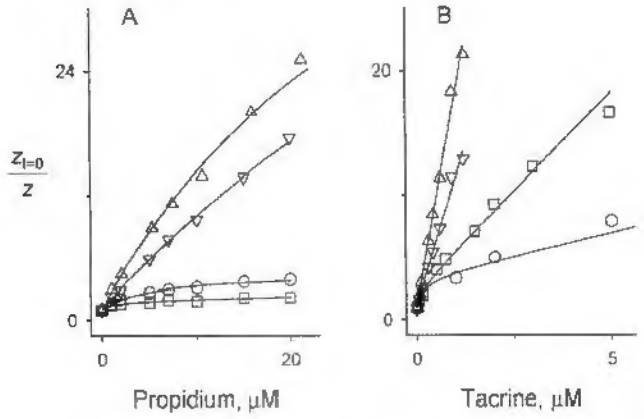


FIG. 3. **Decreased inhibitor affinity following modification of H287C AChE by MTS reagents.** H287C AChE was modified with MTS reagents **II** (▽), **IV** (□), and **V** (○) and isolated as shown in Fig. 1. Aliquots of these AChEs and unmodified H287C AChE (△) were assayed with 20 μM acetylthiocholine at the indicated propidium (*panel A*) or tacrine (*panel B*) concentrations, and relative hydrolysis rate constants ($z_{I=0}/z$) were determined as outlined under "Experimental Procedures." *Panel A*, data for the unmodified enzyme were fitted with Equation 3 to give $K_I = 0.58 \pm 0.05$ μM and $\alpha = 0.016 \pm 0.003$. Data for the modified enzymes were fitted with Equation 4 after fixing K_I at 0.58 μM and α and α_2 at 0.016. *Panel B*, application of Equation 3 to data for the unmodified enzyme gave $K_I = 0.054 \pm 0.002$ μM and $\alpha = 0.002 \pm 0.002$. Data for the modified enzymes were fitted with Equation 4 after fixing K_I at 0.054 μM and α and α_2 at 0. Kinetic parameters obtained from these analyses are given in Table III. Four points from assays with higher tacrine concentrations for AChE modified with **V** (○) were included in the fitting but omitted from *panel B* for clarity.

TABLE III
Inhibition constants for propidium or tacrine with H287C AChEs modified by MTS reagents

H287C modification	Propidium K_{I2}[a]	Tacrine K_{I2}[a]
	μM	
I	2.1 ± 0.3	0.16 ± 0.07
II	1.2 ± 0.8	0.17 ± 0.08
III	16 ± 7	0.11 ± 0.02
IV	54 ± 31	0.67 ± 0.15
V	30 ± 9	4.8 ± 0.3
VI	11 ± 4	14 ± 8

[a] Values of K_{I2} and R_2 were determined from Equation 4 after fixing K_I, α, and α_2 at the values indicated in the legend to Fig. 3. Fitted values of R_2, the fraction of total activity contributed by the modified AChE in the absence of inhibitor, ranged from 0.33 to 0.81 except for AChE modified with **VI**, which gave R_2 values of 0.21 and 0.12. Values of R_2 for a given modified enzyme preparation declined gradually over a period of days, indicating that the modified enzymes were slightly less stable than unmodified H287C. For a given modified enzyme, the decrease in activity between experiments paralleled the decrease in R_2.

intermediate values of K_{app2}, K_{SS2}, and k_{cat2}, between those of H287C AChEs modified with **II** or **V**.

To estimate the extent to which the tethered ligands blocked the P- and A-sites of H287C AChE, inhibition of enzyme activity by propidium and tacrine was examined. Propidium binds specifically to the P-site while tacrine, marketed under the trade name Cognex® for treatment of Alzheimer's disease, is a specific A-site inhibitor. The relative inhibition at low substrate concentrations provided an indication of the affinity of these inhibitors. However, as illustrated in Fig. 2, activity contributions from residual unmodified enzyme were greatest at low substrate concentrations, and quantitative analyses of the inhibition must take these contributions into account. Equation 4 was introduced to allow determination of a K_{I2} for inhibition of a modified AChE in the presence of unmodified AChE showing normal inhibition. Fig. 3*A* illustrates this procedure for propidium inhibition of the four AChEs in Figs. 1 and 2. Inhibition ($z_{I=0}/z$) was measured at seven inhibitor concentrations and in the absence of inhibitor. The plot for unmodified H287C AChE alone showed slight curvature, reflecting a small residual activity when the P-site is saturated with propidium ($\alpha = 0.016$). Plots for the modified AChEs showed more curvature, with inhibition at low concentrations of propidium more influenced by residual unmodified enzyme than those at high concentrations. K_{I2} values for propidium inhibition of H287C modified by **I–VI** were obtained by fitting of these and other plots to Equation 4 (Table III). These K_{I2} values are 2- to 90-fold larger than the K_I value for unmodified H287C, and the magnitude of the increase corresponds to the extent to which a given modification blocked access to the P-site. AChEs modified with **III–VI** are particularly effective in blocking this access, but it is important to note that the K_{I2} values do not increase as the tether in the modification grows from the intermediate lengths in **III** and **IV** to the longer lengths in **V** and **VI**. Plots for tacrine inhibition of the four AChEs in Figs. 1 and 2 are shown in Fig. 3*B*. The plot with unmodified AChE showed no curvature when fitted with Equation 3 ($\alpha = 0.00$). Because tacrine binds to the A-site as a competitive inhibitor, the tacrine-AChE binary complex should have no residual activity as reflected in the zero value for α. The plots for tacrine inhibition of the modified AChEs were curved, again allowing determination of K_{I2} values by fitting with Equation 4 (Table III). However, in contrast to the trend with propidium, the K_{I2} values for tacrine inhibition do not increase substantially until the tether grows from the intermediate length in **III** to the longer lengths in **V** and **VI**. For example, the tacrine binding affinity for H287C AChE modified with **V** decreased nearly two orders of magnitude relative to unmodified AChE.

Molecular Modeling—Tethered ligands offer a distinct advantage in the computational search for compounds that bind with high affinity near the P-site of AChE. Immobilizing one substituent of the ligand significantly reduces the translational degrees of freedom and, hence, the computational time neces-